Signaling Mechanisms and Genetic Regulation of Follicle Cell Maturation and Ovulatory Competence in Drosophila

Elizabeth Knapp, PhD, University of Connecticut, 2020

Abstract

Female fertility is essential for reproduction, and within the ovary requires the proper development of a competent preovulatory follicle and subsequent ovulation. Follicle development into ovulatory competency is intricately orchestrated through successions of temporally coordinated signaling networks within the somatic follicle cells. However, there still remains a lack of understanding of the genetic regulation of this process. The objective of this dissertation is to characterize this process of follicle maturation and ovulatory competency in *Drosophila* to establish its utility as a model system to study folliculogenesis and ovulation. How follicle cells develop in the final stages leading up to ovulation has been very understudied in *Drosophila*. This work focuses on investigating this final maturation and understanding the conserved signaling mechanisms governing it. Firstly, a role for ecdysteroid signaling operating in mature follicle cells to control ovulation was identified. Ecdysteroid signaling had been known to regulate earlier steps of oogenesis, but this study demonstrated another requirement for this signaling in ovulatory competency, reminiscent to progesterone signaling essential for mammalian ovulation. In addition, this work describes the discovery of a novel follicle cell transition occurring in these final maturation stages that is characterized by the dynamic change in expression of follicle cell factors and these findings indicate this follicle cell transition is critical for gaining ovulatory competency. Lastly, a novel role for the NR5A nuclear receptor Ftz-f1 in regulating this final follicle cell maturation process is identified. Furthermore, the mammalian homolog of Ftz-f1, steroidogenic factor 1 (SF-1), was able to replace the function of Ftz-f1 in follicle cell maturation, demonstrating the functional conservation of this process. In summary the work in this dissertation elucidates signaling mechanisms governing follicle cell maturation and ovulatory competency in *Drosophila*. These findings provide enhanced evidence for the conserved nature of these ovarian processes and signifies a strong foundation to utilize *Drosophila* as a model to study folliculogenesis and ovulation.

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Chapter 1 : Introduction

Unresolved Questions in Mammalian Folliculogenesis & Ovulation

Female fertility is an integral half of the equation required for metazoan reproduction. In fact, in some parthenogenetic species, female fertility can serve as the sole component of reproduction, with males not required to produce offspring (Metz and Monroy, 2014). In the United States, approximately 10% of reproductively mature women suffer from infertility (Chandra et al., 2014), and a quarter of these cases are rooted in ovarian disorders such as polycystic ovarian syndrome and premature ovarian insufficiency (Franks, 2008; Rebar, 2000). The underlying causes of these disorders, however, remain unknown. In order to address these questions concerning female infertility, we need to enhance our understanding of the genetic components and molecular mechanisms governing ovarian physiology.

Within the mammalian ovary, two critical processes are required to allow for eventual fertilization of an oocyte—folliculogenesis and subsequent ovulation. The process of folliculogenesis transpires within a functional unit termed the follicle, which consists of an oocyte encapsulated in layers of somatic follicle cells (granulosa cells and theca cells). During folliculogenesis, immature primordial follicles formed near birth must properly progress through several sequential stages (primary, secondary, preantral, antral) to finally yield a fully mature follicle that is competent for ovulation (release of the oocyte from the ovary) (Richards, 2007; Richards and Pangas, 2010). This process of ovulation is triggered by a surge of luteinizing hormone (LH) and can be separated into three main steps—meiotic resumption of the oocyte, expansion of cumulus granulosa cells, and finally follicle rupture (Coticchio et al., 2015; Richards et al., 2015; Russell

and Robker, 2007). During this last step, follicle rupture, the follicular wall breaks down at the follicular apex, and the oocyte is able to extrude from its surrounding follicle and the ovary for release into the oviduct while the residual follicle cells transform into a transient endocrine structure called the corpus luteum. This liberation event requires dynamic structural changes to the follicular epithelium and extensive turnover of the extracellular matrix, which many studies have suggested is regulated by various proteolytic enzymes. The molecular mechanisms and genetic regulation of this system, however, are still poorly understood (Curry and Smith, 2006; Richards et al., 2015). Development and ensuing rupture of the follicle are regulated by a complex network of paracrine, autocrine, and endocrine signaling pathways within the somatic follicle cells. Untangling the functions of these signaling mechanisms will provide us with deeper insight into the physiology of female fertility. One of the most notable types of signal transduction within the ovarian follicle cells essential for female fertility is carried out through the signaling of nuclear hormone receptors (Richards, 2002; Richards and Ascoli, 2018).

Nuclear Hormone Receptor Signaling in the Ovary & Female Fertility

Signaling through the family of nuclear hormone receptors is ubiquitous throughout the animal kingdom, and in humans this family currently consists of 49 identified genes (Robinson-Rechavi et al., 2001). Each member is characterized by a conserved modular structure of domains that allows it to function as a ligand activated transcription factor responsive to various intracellular and extracellular signals (Mangelsdorf et al., 1995; Tsai and O'Malley, 1994). These nuclear receptors are assigned to three classes based on their mode of activation, 1) steroid receptors, 2)

non-steroid receptors, and 3) orphan receptors (Mangelsdorf et al., 1995), and have been further organized into seven subfamilies (NR0-NR6) based on sequence similarity (1999). Of these various nuclear receptors, two groups in particular have shown essential functions within the follicle cells for female fertility—the female sex steroid hormone receptors and the NR5 subfamily of orphan nuclear receptors.

Since the identification of the sex steroid hormone receptors, estrogen receptors (ERs) and progesterone receptors (PRs), over four decades ago, many studies have demonstrated their fundamental roles in regulating female reproductive events (Binder et al., 2015; Toft and Gorski, 1966). Particularly within the ovary, both ER and PR are found to be expressed in the ovarian follicle cells of many species (Durlej et al., 2010; Hild-Petito et al., 1988; Iwai et al., 1990; Tetsuka et al., 1998). Classical signal transduction via ER or PR is activated through their binding to steroid hormone ligands (estrogens and progestogens), which can be synthesized within the follicle cells from cholesterol precursors (Andersen and Ezcurra, 2014; Hanukoglu, 1992; Knobil, 2006; Miller, 1988).

ER signaling is carried out through ERα and ERβ, each a distinct protein encoded by two genes on different chromosomes with almost identical (95%) ligand binding domains (Kuiper et al., 1989). ERα expression is limited to the theca and interstitial cells in rodent ovaries, and granulosa cells of antral follicles in primates, whereas ERβ is highly expressed in the granulosa cells of growing follicles across species (Chiang et al., 2000; Pau et al., 1998; Wang et al., 2000). Multiple rodent studies have indicated these receptors are required for female fertility. ERα knockout mice are anovulatory and exhibit ovaries with pre/small–antral stage follicles and

multiple hemorrhagic cysts, and ERβ knockout mice also exhibit reduced ovulatory capacity and subfertility (Dupont et al., 2000; Krege et al., 1998; Lubahn et al., 1993). Loss of ERα leads to an elevated capacity to synthesize androgens and high levels of estradiol plasma levels (Couse et al., 2003), whereas ERβ knockout mice show decreased ability to synthesize estradiol (Emmen et al., 2005), suggesting that ERα signaling regulates feedback within the gonadotropic signaling loop whereas ERβ is required for follicle maturation past antral stages. However, these receptors also have the potential to heterodimerize, further complicating our understanding of their roles in female fertility (Cowley et al., 1997; Pettersson et al., 1997). Attempts to address this issue through the generation of double knockout mice (αβERKO) have only yielded another layer of complexity. These mice demonstrate a morphological sex reversal with follicles transdifferentiating into structures resembling that of seminiferous tubules of the testis, though, intriguingly, only in sexually mature adults (Couse et al., 1999). Additionally, the mechanisms of ER signaling in the ovary have proven difficult to fully understand due to the fact that ER is expressed in multiple tissues and that few studies have used conditional knockouts of ER specifically in the ovarian follicle cells to assess fertility (Lee et al., 2009; Saunders et al., 1997; Taylor and Al-Azzawi, 2000). More recent studies have shown the majority of ovarian cancer cells exhibit a reduced level of ERβ expression, implicating this receptor may play a crucial role in influencing disease states (Lazennec, 2006). Thus, there is a serious need to further elucidate how ER signaling in the follicle cells regulates follicle development.

Progesterone signaling via the PR has also been shown to be integral for female fertility for decades, with early pharmacological studies inhibiting progesterone

synthesis or PR function able to block ovulation in mice (Loutradis et al., 1991; Tanaka et al., 1991). PR has two isoforms, PR-A and PR-B, which are derived from a single gene via two distinct promoters (Kraus and Katzenellenbogen, 1993). Mice null specifically for PR-A exhibit impaired ovulation and a retention of unruptured preovulatory follicles, while mice null for PR-B ovulate normally, implicating PR-A as the main isoform functioning in female fertility (Mulac-Jericevic et al., 2000, 2003). From rodents to primates, it has been shown that expression of PR is induced by LH in granulosa cells of preovulatory follicles (Choi et al., 2017; Hild-Petito et al., 1988; Robker et al., 2000), and experiments performed with PR knockout mice have shown this LH induced PR expression is essential for eliciting follicle rupture (Lydon et al., 1995, 1996). This role is conserved across species as experiments performed with rhesus monkey follicles demonstrated both pharmacological inhibition of progesterone synthesis and knockdown of the PR in dominant follicles block follicle rupture and ovulation (Bishop et al., 2016; Hibbert et al., 1996). Even though it still remains undetermined through what mechanism PR signaling controls follicle rupture, a number of potential candidates have been recognized as targets of PR signaling. Studies have identified genes that are downregulated in mice null for PR, such as proteases like a disintegrin and metalloproteinase with thrombospondin motifs (*Adamts-1*) and a disintegrin and metalloprotease 8 (*Adam8*) (Robker et al., 2000; Sriraman et al., 2008); these studies hypothesize these proteases could play a role in breakdown of the follicular wall, especially since mice deficient in *Adamts-1* are sub fertile and have trouble ovulating (Mittaz et al., 2004). However, no direct evidence has bolstered these postulations. The ligand-inducible transcription factor peroxisome proliferator-activated

receptor γ (PPARγ), hypoxia inducible factors (HIFs), and the EGF-like growth factors amphiregulin (Areg) and epiregulin (Ereg) have also been identified as targets of PR signaling in the ovary (Kim et al., 2008, 2009; Shimada et al., 2006). Although it seems PR signaling is critical for follicle rupture, more work needs to be done to elucidate this mechanism and to understand the complex genetic regulation involved downstream. Moreover, a majority of these studies were performed with global knockouts of the PR. It is still unclear as to what ovarian defects are caused directly from a loss of PR in the follicle cells or indirectly from crosstalk/feedback among other reproductive tissues. Earlier studies have noted loss of PR causes additional phenotypes in the uterus and mammary glands (Lydon et al., 1995, 1996).

The other nuclear receptor subfamily with a prominent role in female reproduction, NR5, is comprised of two orphan nuclear receptors; steroidogenic factor-1 (SF-1) and liver receptor homolog-1 (LRH-1), both of which are highly expressed in the ovarian follicle cells across species (Falender et al., 2003; Hinshelwood et al., 2003; Mlynarczuk et al., 2013; Sirianni et al., 2002; Takayama et al., 1995). Various mouse models disrupting LRH-1 via conditional knockout, inducible knockdown, or even haploinsufficiency have demonstrated expression of LRH-1 in granulosa cells is required for female fertility (Duggavathi et al., 2008; Gerrits et al., 2014; Labelle-Dumais et al., 2007). These murine studies have revealed LRH-1 is required for several key follicular processes—granulosa cell proliferation, cumulus expansion, follicle rupture, and luteinization—mainly through its regulation of genes associated with the biosynthesis of steroids and prostaglandins (*Star, Cyp11a1, Cyp19a1, Ptgs2*) (Bertolin et al., 2014, 2017; Duggavathi et al., 2008; Gerrits et al., 2014; Labelle-Dumais et al.,

2007; Meinsohn et al., 2017). In contrast, SF-1 is expressed in both the theca and the granulosa cells, and much less is actually known about its role within the ovary. Mice with granulosa cell-specific knockout of SF-1 exhibit severe infertility and fail to properly develop follicles (Pelusi et al., 2008a). It remains to be understood through what mechanism SF-1 promotes follicle maturation and, furthermore, what function SF-1 could play in the theca follicle cells and in follicles during later stages of folliculogenesis. Investigation into the targets of SF-1 has been conducted in various animal models; SF-1 has been shown to regulate the transcription of *Star* in both bovine theca cells and mouse granulosa cells (Murayama et al., 2012; Parker et al., 2002). It also is required to induce expression of *Cyp19a1* in chicken theca cells (Wang and Gong, 2017), and it regulates luteal secretion of inhibin-α in the macaque (Suresh and Medhamurthy, 2012). In addition, recent studies have shown in human granulosa cells that *AMH* (Anti-Mullerian hormone, a hormone critical for controlling the ovarian reserve) is also a target of SF-1 (Jin et al., 2016).

Thus, for over two decades it has been established that signaling through both these groups of nuclear hormone receptors is essential for proper follicle development and ovulation. Despite the imperative function these nuclear receptors serve within the ovarian follicle cells, there still remain many unanswered questions as to their mechanism of action and genetic regulation. Further study of these nuclear receptors and their functions within the context of the ovary will provide us with a more detailed insight into the complex regulation of folliculogenesis and ovulation, improving our understanding of female fertility as a whole.

Need for a Model to Study Folliculogenesis & Ovulation

Even though follicle cell expression of these nuclear hormone receptors is essential for proper follicle development and ovulation, there remain many unanswered questions as to their mechanism of action and genetic regulation. This is in part due to some of the inherent limitations of mammalian models, such as the previously discussed issues of genetic redundancy and a lack of studies performed to precisely target follicle cells throughout specific developmental stages. In mammals, untangling the multiple stage-specific roles of these nuclear hormone receptors and identifying their downstream targets and mechanisms of actions within the ovarian follicles becomes tedious and complex. Additionally, there are limitations in the current techniques used to probe follicle maturation in mammalian models. Previously, follicle maturation was analyzed using ultrasonography. But ultrasonography is not a reliable method to observe younger, smaller stages of follicle development. Recent studies have developed a new method to track follicle development using ovarian tissue cultures and time-lapse analysis (Komatsu and Masubuchi, 2017; Komatsu et al., 2015), however, while this technique does yield a more detailed analysis, it takes a four week period, requiring an entire month to yield results. Thus, there is a need for a more efficient, simpler, and genetically tractable model system to further study the signaling mechanisms governing follicle development and ovulation.

The common fruit fly, *Drosophila melanogaster,* is an ideal model organism to serve this need. They are inexpensive, reproduce efficiently, have less overall genetic redundancy, come equipped with an abundance of excellent genetic tools, and have a high degree of conservation—approximately 75% of disease-related genes in humans

have functional orthologs in *Drosophila* (Bier, 2005; Reiter et al., 2001). Especially in the context of studying fertility, *Drosophila* are an attractive model, owing to their enhanced level of fecundity combined with their striking similarities in reproductive anatomy and function to mammals. The essential ovarian processes of folliculogenesis and ovulation seem to be specifically conserved, and newly developed experimental assays to probe these processes in *Drosophila* make it a premier model to further study the genetic regulation of female fertility (Knapp et al., 2018). Lastly, key signaling pathways, like those through nuclear hormone receptors, are also conserved in *Drosophila*, and both signaling through steroid nuclear hormone receptors and the orphan NR5 family have been extensively characterized in *Drosophila (King-Jones and Thummel, 2005)*. In conclusion, the intent of this dissertation will be in part to demonstrate the utility of *Drosophila* as a model to study the genetic regulation and signaling mechanisms required for folliculogenesis and ovulation and to further elucidate conserved mechanisms required for female fertility across species.

Drosophila Female Reproductive System Anatomy

The female reproductive system in *Drosophila* shares many similarities to that of mammals; a pair of ovaries are connected to lateral oviducts, which join into a common oviduct that leads into the uterus where internal fertilization can take place (Spradling, 1993). Attached to the uterus via ducts are two pairs of specialized exocrine glands (the spermathecae and parovaria), these are comprised of secretory cells (analogous to the mammalian secretory cells lining the oviduct epithelium) and are required for female fertility and sperm storage (Allen and Spradling, 2008; Anderson, 1945; Schnakenberg

et al., 2011; Sun and Spradling, 2013). Additionally, the female reproductive system in *Drosophila* is extensively innervated by octopaminergic neurons (Middleton et al., 2006) akin to the adrenergic innervations of mammalian ovaries (Aguado, 2002), and in both systems it seems adrenergic signaling plays a critical role in regulating reproductive physiology (Barria et al., 1993; Blum et al., 2004; Deady and Sun, 2015; Lim et al., 2014; Rodríguez-Valentín et al., 2006). Studying ovarian physiology in *Drosophila* is also aided by the facts that development of a reproductively mature female fly takes less than a week after eclosion and that the ovary is the largest organ in the female fly. These properties combine to provide an efficient and easy system in which to manipulate and analyze ovarian processes like folliculogenesis and ovulation.

Folliculogenesis & Ovulation in Drosophila

Similar to those of mammals, the ovarian follicles in *Drosophila* are comprised of an oocyte encased in a layer of somatic follicle cells and require a follicle development process to yield mature preovulatory follicles. In *Drosophila,* the process of folliculogenesis is divided into 14 distinct maturation stages, with series of developing follicles organized into approximately 16-20 assembly lines called ovarioles (King, 1970; Spradling, 1993). Thus, within one ovary, multiple follicles can be efficiently generated simultaneously, and an array of sequentially developing follicles can be analyzed, creating an ideal system in which to study follicle development. This process begins in the anterior tip of the ovary in a region called the germarium. Here, germline stem cells (GSC) reside and undergo asymmetric division to yield another GSC and a differentiated cystoblast. The cystoblast then undergoes four mitotic divisions, each with

incomplete cytokinesis, resulting in a 16-cell germline cyst with cells interconnected to one another through intercellular cytoplasmic bridges called ring canals (Margolis and Spradling, 1995; Spradling et al., 1997), a process that seems to also be conserved in mammals (Pepling and Spradling, 1998). Only one of the cells will be specified as the oocyte and is positioned at the posterior; the remaining 15 cells become nurse cells situated anteriorly, and all are then encapsulated by a layer of somatic follicle cells to form a stage-1 follicle. The follicle then progresses through 14 stages of maturation, where the oocyte progressively grows whilst the encapsulating follicle cell layer undergoes a series of tightly coordinated developmental and morphological processes. Analogous to mammals, the follicle cell epithelium plays a critical role in mediating various intracellular signaling pathways to coordinate proper development and yield a competent preovulatory follicle. Over the past few decades, a substantial amount of work has focused on the signaling mechanisms and genetic factors governing follicle cell development during early stages of oogenesis, yet very little has been done to investigate follicle cells in the final stages leading up to the stage-14 preovulatory follicle.

Follicle Cell Transitions and Functions in Early Stages

The encapsulating layer of somatic follicle cells serves essential functions throughout follicle development and is required for establishing the main body axes of the future embryo, synthesizing the protective eggshell, generating specialized eggshell structures, and eliciting follicle rupture and ovulation. Throughout the early stages of follicle development, follicle cells experience two critical transitions that have been well characterized—the mitotic to endocycle transition (M/E) and the endocycle to gene

amplification transition (E/GA) (Calvi et al., 1998; Edgar and Orr-Weaver, 2001). In the beginning of folliculogenesis from stage 1 to 6, follicle cells are first in a state of mitosis and carry out approximately 8 or 9 rounds of archetypal mitotic division to produce ~1000 follicle cells in the span of about 30 hours (Nystul and Spradling, 2010; White et al., 2009). Signaling through both the Hedgehog and c-Jun N-terminal kinase pathways has been shown to promote the mitotic cycle during these stages by regulating expression of various cyclins and cyclin-dependent kinases (Forbes et al., 1996; Jordan et al., 2006; Zhang and Kalderon, 2001). Additionally, expression of the homeodomain transcription factor Cut in follicle cells from stage 1 to 6 is required for mitosis through its promotion of Cyclin A expression (Sun and Deng, 2005).

From stage 6 to stage 7, follicle cells undergo their first characterized transition as they shift out of the mitotic cycle and into a state of endocycle. From stage 7 to stage 10A, follicle cells undergo three rounds of endocycle where their genomic DNA is duplicated without cell division, yielding polyploid follicle cells with 16 copies of genomic DNA (Edgar and Orr-Weaver, 2001; Lilly and Spradling, 1996). Transition into endocycle is regulated through Notch signaling at the apical surface of follicle cells, which is activated by germline expression of Delta in stage-7 follicles (Deng et al., 2001; Shcherbata et al., 2004; St. Johnston, 2001). Notch signaling at this transition is required to activate the zinc-finger transcription factor Hindsight (Hnt), which functions to inhibit hedgehog signaling and repress expression of Cut. Loss of Hnt during these stages causes follicle cells to delay entry into endocycle, while ectopic expression of Hnt before stage 7 is sufficient to push follicle cells into endocycle prematurely (Sun and Deng, 2007).

The next characterized follicle cell transition occurs at stage 10A to stage 10B as follicle cells shift out of endocycle into a state of gene amplification. From stage 10B to 13, genes encoding chorion proteins necessary for eggshell synthesis are amplified through continuous re-replication without a resting gap phase (Calvi et al., 1998). Further studies have shown this process can be subdivided into two phases. First, from stage 10B to 11 the origin recognition complex (ORC) localizes to these chorion gene clusters and DNA replication is initiated. Then, from stage 12 to 13 ORC is lost from chorion origins, and only existing replication forks progress outward in this elongation-only phase (Claycomb et al., 2002). The E/GA switch requires both the downregulation of Notch signaling and an upregulation of steroid signaling through the ecdysone receptor (EcR) to promote expression of the zinc finger transcription factor Ttk69 (Sun et al., 2008). Loss of Ttk69 inhibits gene amplification, and premature upregulation of Ttk69 is sufficient to induce precocious exit from endocycle. Additionally, by stage 10B Ttk69 downregulates expression of Hnt and re-upregulates expression of Cut in follicle cells. Overall, these key transitions exhibited by follicles cells during early oogenesis are coordinated and marked by distinct molecular patterns of receptors and transcription factors, however it is still yet to be investigated what genetic profiles follicle cells express in the following stages of folliculogenesis.

As mentioned previously, follicle cells proliferate until stage 6, and after they cease division, they start to differentiate into specific cells types in order to pattern the main body axes and specialized eggshell structures. The anterior-posterior axis is first established, starting in stage 5 when a pair of specialized follicle cells (polar cells) at both ends of the follicle stop division early. These polar cells stimulate a graded

activation of the Janus kinase (JAK) pathway along the anterior posterior axis, with different levels of JAK/STAT activity specifying distinct anterior follicle cell fates and the integration of signaling from JAK/STAT and epidermal growth factor receptor (EGFR) pathways specifying posterior follicle cell identities (Denef and Schüpbach, 2003; Fregoso Lomas et al., 2016; Gonzalez-Reyes and Johnston, 1998; Silver and Montell, 2001; Xi et al., 2003). Next, in stage-8 follicles, the dorsal-ventral axis is also established through EGFR signaling, initiated in follicle cells contacting the newly moved oocyte nucleus at the dorsal anterior corner (Neuman-Silberberg and Schüpbach, 1993; Queenan et al., 1997). Multiple cascades of EGFR signaling combined with activation of transforming growth factor-β pathways via the morphogen Decapentaplegic (Dpp) are required to specify groups of dorsal-anterior follicle cells that will eventually serve as the basis for specialized egg shell structures such as the dorsal appendages (respiratory tubes) and the operculum (the larval escape hatch) (Deng and Bownes; Morimoto et al., 1996; Twombly et al., 1996; Wasserman and Freeman, 1998). Patterning of these structures also requires key migratory events prior to eggshell synthesis such as border cell migrations at stage 9, and centripetal cell migration at stage 10B (Margaritis et al., 1980; Montell, 2001; Montell et al., 1992). A great deal of research has been performed to understand the complex signaling governing dorsal appendage morphogenesis. After their cell fate has been specified at stage 10B, subsets of dorsal-anterior follicle cells begin to wrap into a tube formation in stage 11, and, at stage 12, these tubes then elongate and extend anteriorly while secreting eggshell components into their lumens. Finally, from stage 13 to 14, the anterior paddle structures mature and dorsal appendage formation is complete (Dorman et al., 2004;

French et al., 2003; Rittenhouse and Berg, 1995; Suzanne et al., 2001). Aside from this subset of dorsal-anterior follicle cells, however, not much has been studied pertaining to the rest of the follicle cells in the final stages of folliculogenesis as they progress into a preovulatory state.

Follicle Rupture & Ovulatory Competency

Within the past few years, a role and identity has emerged for stage-14 follicle cells in the context of ovulation. Recent work has demonstrated that, akin to mammalian ovulation, ovulation in *Drosophila* requires a follicle rupture event where the oocyte is expelled out of its encapsulating follicle cell layer and ovulated out of the ovary into the oviduct, with residual follicle cells forming a corpus luteum-like structure in the ovary. Follicle rupture is triggered through octopaminergic signaling, with Octopamine (OA) activating its receptor Oamb on stage-14 follicle cells. Downstream of Oamb signaling, different enzymes are activated to facilitate this rupture process, such as matrix metalloproteinase 2 (Mmp2) and NADPH oxidase (Nox) (Deady and Sun, 2015; Deady et al., 2015; Li et al., 2018). Mmp2 is required for proteolytic degradation of posterior follicle cells to initiate the rupture process, and Nox activity in main-body follicle cells is essential for generation of reactive oxygen species (ROS) necessary for ovulation. Mammalian studies have also implicated the requirement of proteases like Mmps and ROS production for ovulation, suggesting this process is fairly conserved across species (Curry and Osteen, 2003; Shkolnik et al., 2011). In addition to the discovery of this conserved process, such studies have also developed novel *ex vivo* assays to test follicle rupture, protease activity, and ROS production (Knapp et al., 2018; Li et al., 2018), allowing researchers to probe the physiology of just the preovulatory follicle

without complications from additional tissues and enabling both genetic and pharmacological manipulation of preovulatory follicle cells.

With these studies, we have also begun to characterize the identity of a stage-14 follicle cell, and we observe that the expression of these critical components (Oamb, Mmp2, Nox) for follicle rupture are specifically upregulated in stage-14 follicle cells. It therefore seems likely that follicle cells experience another coordinated transition in the final stages of folliculogenesis as they shift into a state competent for follicle rupture and ovulation. Overall, the significance of these recent findings about follicle rupture in *Drosophila* and the lack of knowledge concerning late follicle cell development into stage 14 leave unresolved questions about what signaling mechanisms regulate the development of follicle cells into ovulatory competency and what additional factors are important in mature follicle cells for follicle rupture and ovulation.

Nuclear Hormone Receptor Signaling Homologs in Drosophila

As previously stated, in addition to their conserved ovarian anatomy and physiology, *Drosophila* also utilize nuclear hormone receptors. Further investigation into their potential roles within *Drosophila* ovaries could yield insight into the genetic regulation of female fertility. The *Drosophila* genome contains 21 nuclear-receptor genes (Adams et al., 2000), with members of this nuclear-receptor family characterized by the presence of a highly conserved N-terminal DNA-binding domain and a less conserved C-terminal ligand binding domain joined by a flexible hinge region. These combined receptor and DNA-binding elements allow for a signal transduction to directly affect target gene transcription (King-Jones and Thummel, 2005).

Steroid signaling through the ecdysteroid pathway is one of the most classically studied forms of nuclear hormone receptor signaling in *Drosophila*. The steroid signaling molecule twenty-hydroxyecdysone (commonly referred to as ecdysone) activates a heterodimeric nuclear receptor complex comprised of the ecdysone receptor (EcR) and its partner ultraspiracle (USP), which is then able to bind to specific promoter sequences (ecdysone response elements) to regulate expression of various genes (Koelle et al., 1991; Thummel, 1996; Yao et al., 1993). Ecdysone derives its name for its monumental role in triggering ecdysis in arthropods and has been well studied for over 50 years for its role in regulating development and metamorphosis in *Drosophila*.

Prior to adulthood, *Drosophila* proceed through various developmental stages; starting with embryogenesis, followed by three larval stages, ending with puparium formation and metamorphosis, each of which is stimulated by a pulse of ecdysone. Initial studies of puffing patterns in polytene chromosomes from larval salivary glands demonstrated ecdysone induces gene expression in a temporally patterned manner (Ashburner, 1972, 1974; Ashburner and Richards, 1976; Richards, 1976), with subsequent work showing ecdysone triggers cascades of gene expression with many direct targets being other nuclear hormone receptors. This ecdysone signal is transduced into waves of gene expression and can be divided into four temporal profiles; early genes, early late genes, mid prepupal genes, and late prepupal genes (Huet et al.). The early genes are rapidly and directly induced by high levels of ecdysone and include the nuclear hormone receptor E75 as well as the transcription factors Broad-Complex and E74 (DiBello et al., 1991; Segraves and Hogness, 1990; Thummel et al., 1990). The early late genes, including various nuclear receptors such

as DHR3, DHR39, and E78, exhibit a delayed expression and are induced partially by ecdysone signaling and partially by other protein synthesis (Horner et al., 1995; Stone and Thummel, 1993). Once ecdysone titers have subsided, mid prepupal genes are then expressed, only one of which has been identified (the nuclear hormone receptor Ftz-f1) and is required for competency of the following ecdysone pulse and induction of late-prepupal genes (Lavorgna et al., 1993; Richards, 1976).

Analogous to steroid signaling in mammals, ecdysteroid signaling also plays a prominent role in the adult ovaries of *Drosophila*. Akin to its pulse like nature of regulation throughout development, ecdysone signaling regulates developmental transitions throughout folliculogenesis. In early stages of oogenesis ecdysone signaling within the germarium is required for GSC proliferation and maintenance, as well as for niche formation and cystoblast differentiation (Ables and Drummond-Barbosa, 2010; König et al., 2011). In addition, throughout mid-oogenesis ecdysone signaling is required for; follicles to progress past previtellogenic stages, follicle cell migrations, the transition into gene amplification and eggshell production, as well as oocyte lipid accumulation (Boyle and Berg, 2009; Buszczak et al., 1999; Carney and Bender, 2000; Sieber and Spradling, 2015; Sun et al., 2008; Terashima et al., 2005). A potential role for ecdysone signaling in late stages of oogenesis have been slightly hinted at, but never actually investigated. For example, early experiments with temperature sensitive EcR mutants only focused on assessing defects associated with follicle maturation in vitellogenic stages (stages 8-10), however researchers noted that these EcR mutants also exhibited an increased retention of stage-14 follicles, indicating an ovulation defect (Carney and Bender, 2000). Additionally, studies using transgenic reporter gene

constructs have demonstrated activity of EcR is mostly restricted to follicle cells, with high levels of activity seen at stage-10 that subsided until activity increased again in stage-14 follicle cells (Hackney et al., 2007). However, no role for ecdysone signaling in the preovulatory follicle had been investigated. Akin to mammalian steroid signaling, it seems likely ecdysone signaling could function in the ovarian follicle cells to regulate competency for ovulation as well.

Similar to mammals, the *Drosophila* genome also encodes two members belonging to the NR5A subfamily of nuclear hormone receptors; Ftz-f1 and HR39 (Ayer et al., 1993a; Ueda et al., 1990), both of which share high sequence similarity and can bind to identical target sequences *in vitro* (Ayer et al., 1993a; Ohno et al., 1994). There seems to be a high degree of conservation across species within this subfamily, with previous work demonstrating defects caused by loss of Ftz-f1 or HR39 can be either partially or fully rescued with their mammalian homologs (Lu et al., 2013; Sun and Spradling, 2012). Both Ftz-f1 and HR39 are expressed downstream of ecdysteroidal signaling during the different stages of development and metamorphosis, with HR39 expression peaking during the high titer pulses of ecdysone and inversely Ftz-f1 only expressed in periods between the ecdysone pulses (Horner et al., 1995; Sullivan and Thummel, 2003). More recent work has demonstrated that proper development of the secretory glands (spermathecae and parovaria) of the female reproductive tract is regulated by HR39 (Allen and Spradling, 2008; Sun and Spradling, 2012), and expression of HR39 in the adult secretory cells of these glands plays a critical role in female fertility to regulate both sperm storage and ovulation (Sun and Spradling, 2013). In contrast, much less is known about the role of Ftz-f1 in the adult, with the majority of

studies focusing on the function of Ftz-f1 during development and metamorphosis. The tight temporally coordinated expression of Ftz-f1 is required for successful transition through embryogenesis, larval molting, and pupation as it has been to shown function as a competency factor for the subsequent ecdysone pulse (Agawa et al., 2007; Woodard et al., 1994; Yamada et al., 2000). Despite the conservation of Ftz-f1 and the known importance of ecdysteroid signaling in adult ovaries, it has yet to be investigated if Ftz-f1 functions within the adult to regulate aspects of female fertility akin to its mammalian homologs.

Need for a Method to Study Follicle Cell Maturation Regulating Ovulatory Competency

When considering the reproductive efficiency, wealth of genetic tools, similarities in ovarian and follicular anatomy, conservation of nuclear hormone receptors, and recent studies demonstrating the conserved mechanisms of follicle rupture and ovulation, *Drosophila* are an outstanding model system to investigate folliculogenesis and ovulation. However, relatively little is known about follicle cells in the final stages of folliculogenesis, with the focus of most ovarian studies in *Drosophila* stopping at stage 11. Thus, there is a need to characterize how follicle cells progress after stage 11 and understand how they differentiate into a state primed for follicle rupture and ovulation by stage 14. The goal of this body of work is to elucidate what additional follicle cell factors are required for ovulatory competency and how are they regulated in these final stages of follicle development. In particular, these investigations center around the potential conserved role of nuclear hormone receptor signaling (through both ecdysteroidal signaling & NR5 Ftz-f1 signaling) in these follicle cells, considering these homologous

signaling pathways in mammalian follicle cells are essential for folliculogenesis and ovulation. In summary, this dissertation will characterize how follicle cells mature in the final stages of folliculogenesis and demonstrate that competency for follicle rupture and ovulation is regulated through conserved signaling mechanisms, thus further supporting the utility of *Drosophila* as a model to study folliculogenesis and ovulation.

Chapter 2 : Steroid Signaling in Mature Follicles is Important for Drosophila Ovulation

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Introduction

Ovulation is crucial for reproduction and requires the proteolytic breakdown of the ovarian follicle for releasing a fertilizable oocyte (Curry and Osteen, 2003; Takahashi et al., 2013). This process consists of a complex series of events tightly coordinated by a network of paracrine and endocrine signals, most notably in mammals by steroid progesterone signaling, in mature follicles (Espey and Richards, 2006; Kim et al., 2009; Fan et al., 2012). Pharmacological inhibition of progesterone synthesis or genetic disruption of the progesterone receptor results in an ovulation failure due to a lack of follicle wall proteolysis or follicle rupture (Lydon et al., 1995; Hibbert et al., 1996). The signaling mechanism by which progesterone regulates a spatiotemporal proteolysis leading to a successful follicle rupture is largely unknown.

Recent work in *Drosophila* has demonstrated some striking similarities between *Drosophila* and mammalian ovulation. *Drosophila* ovaries are organized into ovarioles, which consist of a string of egg chambers developing through 14 different stages into mature follicles (stage-14) (Spradling, 1993). Like mammals, mature oocytes in *Drosophila* are wrapped in a layer of somatic follicle cells. During ovulation, posterior follicle cells are partially broken down to allow oocytes to be released into the oviduct, while the residual follicle cells develop into a corpus luteum (Deady et al., 2015). This follicle rupture also relies on proteolytic enzymes, like Matrix metalloproteinase 2 (Mmp2), which is specifically expressed in the posterior follicle cells of stage-14 egg chambers (Deady et al., 2015). Mmp2 activation is stimulated by the monoamine octopamine (OA), which is equivalent to norepinephrine in mammals and is essential for ovulation (Monastirioti, 2003; Monastirioti et al., 1996). OA binds to its receptor Oamb

(Octopamine receptor in mushroom body) in mature follicle cells to induce an intracellular calcium rise, Mmp2 activation, and follicle rupture (Deady and Sun, 2015). *Drosophila* ovulation is also regulated by multiple ovarian extrinsic factors, including secretions from oviduct (Lim et al., 2014), female reproductive glands (Sun and Spradling, 2013), and male accessory glands (Rubinstein and Wolfner, 2013; Yapici et al., 2008). However, a role for steroid signaling in *Drosophila* ovulation has never been established.

In *Drosophila*, the major steroid hormones are ecdysteroids, including ecdysone (E) and the most biologically active form 20-hydroxyecdysone (20E). Many enzymes involved in the ecdysone biosynthesis pathway have been identified, including Phantom (Phm), Disembodied (Dib), Shadow (Sad), and Shade (Shd), which carry out the final four steps of 20E synthesis (Chavez et al., 2000; Warren et al., 2002; Petryk et al., 2003; Warren et al., 2004). In addition, enzymes encoded by *spook* (*spo*) and *shroud* (*sro*) act more upstream for chemical reactions designated as the black box (Niwa et al., 2010; Ono et al., 2006). Ecdysteroids signal through a heterodimeric nuclear receptor complex comprised of Ultraspiracle (Usp) and Ecdysone receptor (EcR) (Koelle et al., 1991; Yao et al., 1992). The latter has three alternative splicing isoforms (EcR.A, EcR.B1, and EcR.B2), which vary at their N-terminal regions (Talbot et al., 1993). All three isoforms have identical DNA binding and ligand binding sequences, but differ in transcriptional activation and repression (Hu et al., 2003). Ecdysteroids have long been known to play essential roles in developmental transitions, such as larval molting and metamorphosis (Riddiford et al., 2000; Thummel, 1996). In contrast, the roles of ecdysteroids in adult physiology have just started to emerge (Bernardi et al., 2009;

Ameku and Niwa, 2016; Ables and Drummond-Barbosa, 2010; Buszczak et al., 1999; Carney and Bender, 2000; Hackney et al., 2007; König et al., 2011; Sieber and Spradling, 2015; Sun et al., 2008; Terashima et al., 2005; Domanitskaya et al., 2014).

In this study, we characterized the role of ecdysteroid signaling in late oogenesis, mainly as a key component in mediating ovulation. The enzyme Shd, which converts E to 20E, is dramatically upregulated in mature follicle cells. Disruption of ecdysteroid signaling in these follicle cells blocked OA-induced follicle rupture and ovulation. Ecdysteroid signaling is essential for proper activation of Mmp2 enzymatic activity. Our work thus demonstrates the novel role of ecdysteroid signaling in *Drosophila* ovulation, reminiscent to progesterone signaling in mammalian ovulation.

Results

E-20-monooxygenase Shade is required in mature follicle cells for ovulation

Shd, the E-20-monooxygenase, converts E to 20E to elicit steroid signaling during early development (Petryk et al., 2003). To investigate the role of steroid signaling in ovulation, we characterized the expression pattern of Shd in late oogenesis. Shd is enriched in follicle cells at stage 10 (Fig 2.1A) and downregulated to an undetectable level at stage 13 (Fig 2.1B). At stage 14, Shd is dramatically upregulated in all follicle cells (Fig 2.1C). This prominent upregulation of Shd in mature follicle cells prompted us to investigate its role in ovulation. With *R44E10-Gal4* driving *UAS-shd*^{RNAi} expression in mature follicle cells (Deady and Sun, 2015), *shd* expression is severely knocked down in mature follicles but normal in early egg chambers (Fig 2.1D and Fig 2.2). Female flies with *shd* knockdown laid significantly fewer eggs in two days (Fig 2.1E). This egg-laying

defect can be completely rescued by ectopic expression of *shd* in mature follicle cells (Fig 2.1F). A similar egg-laying defect was observed when *shd* was knocked down by *R47A04-Gal4* (Fig 2.3), another Gal4 line overlapped with *R44E10-Gal4* in mature follicle cells (Deady et al., 2015). These data suggest that Shd functions in mature follicle cells to regulate egg laying.

The egg laying consists of ovulation (releasing eggs from follicles), egg transport through the oviduct, and oviposition (releasing eggs from the uterus to the external environment). To determine which steps are altered when *shd* is knocked down, we investigated the distribution of eggs in the female reproductive tract (Table 1) and calculated the average time of each egg spent in the ovulation, in the oviduct, or in the uterus. When *shd* was knocked down, the time required for ovulation was significantly longer than controls (Fig 2.1G and Table 1). The four-fold increase in ovulation time could not be solely explained by the slight reduction of mature follicles in the ovaries (Fig 2.1H), but rather by an ovulation defect (see below). These data suggest that upregulation of Shd in mature follicle cells is essential for normal ovulation.

Shade synthesizes 20-hydroxyecdysone to regulate follicle rupture

Since follicle rupture can be recapitulated by *in vitro* culture of mature follicles with octopamine stimulation (Deady and Sun, 2015), we performed the *in vitro* assay to determine whether *shd*-knockdown follicles (*shd* follicles) are competent for OA-induced follicle rupture. Consistent with our previous results (Deady and Sun, 2015), 54% of control follicles had ruptured with a three-hour OA stimulation (Fig 2.4A and Fig 2.4C). In contrast, less than 15% of *shd* follicles had ruptured (Fig 2.4B-C). The decreased rate of follicle rupture can be fully rescued by ectopic expression of *shd* (Fig 2.4D-F),

consistent with the egg-laying results (Fig 2.1F). Altogether, these data indicate that Shd in mature follicle cells is important for follicle rupture/ovulation. It is interesting to note that knocking down upstream enzymes for E synthesis in mature follicle cells only caused a moderate reduction of egg-laying number but no statistically significant defect in follicle rupture/ovulation (Fig 2.1E, G, Fig 2.4C, and Table 1). Unlike Shd, Phm was highly enriched in stage-13 follicle cells (Fig 2.5A-B). This likely suggests that these upstream enzymes functions in younger follicle cells for E synthesis.

To determine whether Shd regulates follicle rupture through synthesizing 20E, we set to rescue the rupture defect of *shd* follicles with exogenous 20E. Interestingly, supplementing with 20nM of 20E, the typical concentration found in hemolymph (Handler, 1982), can significantly increase the OA-induced rupture to about 40% in *shd* follicles in three hours; however, 100nM of 20E did not elicit a better rescue, if not worse (Fig 2.6A-C). Neither did addition of 20E affect OA-induced follicle rupture in control follicles (Fig 2.6A-B), nor was 20E alone sufficient to induce follicle rupture (Fig 2.6D). Since ecdysone signaling controls transcription, we reasoned that extending the culture period may have an improved rescue effect. Thus, we performed similar experiments and examined the follicles at the end of a six-hour culture. Consistent with this idea, we did observe an increased rupture rate in *shd* follicles to more than 50% with 20nM 20E supplement (Fig 2.4H-I and Fig 2.6E). In contrast, supplementing with 20nM of E did not elicit any rescue effect (Fig 2.4G and Fig 2.4I). Altogether, the rescue of the rupture defect in *shd* follicles with 20E but not E support the notion that Shd functions in mature follicle cells to convert E to 20E to regulate follicle rupture.

EcR is required in mature follicle cells for follicle rupture/ovulation

In *Drosophila*, ecdysteroids signal through the EcR/Usp complex. To examine the role of EcR in ovulation, we utilized a combination of temperature sensitive (ts) and null alleles of *EcR* (*EcR*^{ts} in short) (Carney and Bender, 2000), which allows follicles to develop into stage 14 normally when shifted to restrictive temperature after adult eclosion. *EcR*^{ts} females with such treatment had a significant reduction in the egg-laying number and a slight increase in mature follicles in ovaries (Fig 2.7A-B). In addition, *EcR*^{ts} females displayed a seven-fold increase in ovulation time compared to control females (Fig 2.7C and Table 1), supporting a role for EcR in ovulation.

To confirm that EcR indeed functions to regulate follicle rupture like Shd, we isolated mature follicles from *EcR*^{ts} females and performed *in vitro* follicle rupture. Mature follicles from *EcR*^{ts} females showed a significant reduction in follicle rupture in comparison to controls when stimulated with OA (Fig 2.7D-F). These results demonstrate a role for EcR in follicle rupture/ovulation.

To determine whether EcR directly functions in mature follicle cells to regulate follicle rupture, we specifically disrupted EcR function in these cells using a dominant negative form of *EcR* (*EcR.B1*^{DN}), which binds to ecdysone-responsive genes without activating their expression (Cherbas et al., 2003), and tested *in vitro* follicle rupture. Mature follicles from two different *EcR.B1*^{DN} mutants ruptured at only a third of the rate or less of control follicles when exposed to OA (Fig 2.7G-I). This indicates a requirement of EcR in mature follicle cells for proper follicle rupture.

EcR.B2 but not other isoforms regulates follicle rupture

The *EcR* gene encodes three protein isoforms (EcR.A, EcR.B1, and EcR.B2), with EcR.B2 having the shortest N-terminus (Talbot et al., 1993). To determine which isoform functions in mature follicle cells, we examined the EcR expression using isoform-specific antibodies (Talbot et al., 1993). EcR.B1 is highly enriched in follicle cell nuclei from stage 10 to stage 12 but downregulated to an undetectable level from stage 13 to 14 (Fig 2.8A-B and Fig 2.9A). EcR.A has a similar expression pattern as EcR.B1 and is also not detected in stage-14 follicle cells (Fig 2.8C-D and Fig 2.9B). In contrast, the antibody recognizing all three isoforms detected EcR expression in follicle cells throughout oogenesis including stage 14 (Fig 2.8E-F and Fig 2.9C). These data imply that EcR.B2 is likely the isoform expressed in mature follicle cells. Unfortunately, no EcR.B2-specific antibody has been generated to allow us to detect its expression pattern directly.

To determine whether EcR.B2 functions in follicle rupture, we first utilized isoform-specific RNAi lines to knockdown *EcR* in mature follicle cells. *EcR.A* or *EcR.B1* knockdown did not block OA-induced follicle rupture (Fig 2.8G and Fig 2.10A). No *RNAi* line targeting EcR.B2 is available. In contrast, *RNAi* targeting an EcR common region (knocking down all three isoforms) showed a decrease in follicle rupture rate (Fig 2.8G and Fig 2.10B). This result suggests that EcR.B2 or a combination of isoforms is required to regulate follicle rupture.

To explicitly test whether 20E signals through the EcR.B2 isoform or a combination of isoforms to regulate follicle rupture, we aimed to rescue the rupture

defect in *EcR.B1*^{DN} mutants by overexpressing the individual EcR isoforms in mature follicle cells. Similar experiments have previously been performed and found that three EcR isoforms are interchangeable to support multiple organ development, while only EcR.A supports development of wing margins and EcR.B2 functions in larval epidermis and border cell migration (Cherbas et al., 2003). Interestingly, we found that overexpression of EcR.B2, but not EcR.B1 or EcR.A, completely rescued the OA-induced rupture defect seen in *EcR.B1*^{DN} follicles (Fig 2.8H-J). In addition, overexpression of EcR.B2 alone in mature follicle cells did not interfere with OA-induced follicle rupture, while overexpression of EcR.B1 or EcR.A significantly decreased OA-induced rupture to less than 20% (Fig 2.8H). Thus, the normal downregulation of EcR.B1 and EcR.A in mature follicle cells is essential to allow proper ecdysteroid signaling for follicle development and rupture. In summary, our results demonstrate the requirement for EcR in mature follicle cells to regulate follicle rupture, and specifically, only the EcR.B2 isoform is sufficient to function in this process.

Ecdysteroid signaling regulates follicle rupture by promoting Mmp2 activation

OA activates the Oamb receptor in mature follicle cells, which sequentially leads to an increase of intracellular calcium, activation of Mmp activity, and follicle rupture (Fig 2.11A) (Deady and Sun, 2015). To determine whether ecdysteroid signaling interferes with this pathway to regulate follicle rupture, we measured the activation of Mmp2 in control and steroid-defective follicles with *in situ* zymography. Consistent with our previous report (Deady and Sun, 2015), about 80% of control follicles showed gelatinase activity at their posterior end after OA stimulation for three hours or six hours; however, only 40% of *shd* follicles showed gelatinase activity (Fig 2.11B,D, and Fig

2.12A-C). This reduction of gelatinase activity can be partially rescued by supplementing 20E in the culture medium (Fig 2.11C-D). In addition, *EcR*^{ts} follicles also showed reduced gelatinase activity in comparison to controls after OA stimulation (Fig 2.12D-F). These data suggest that follicles lacking ecdysteroid signaling are unable to efficiently activate Mmp2 in response to OA stimulation, which may explain their poor response to OA-induced follicle rupture and ovulation.

To find out where ecdysteroids interfere with the OA/Oamb-Mmp2 pathway, we examined the Mmp2 expression level with an *Mmp2::GFP* fusion reporter and qRT-PCR. Mmp2 expression is not affected by ecdysteroid signaling at either the mRNA or protein level (Fig 2.9E and Fig 2.12G-I). In addition, *Oamb* mRNA expression is also normal when ecdysteroid signaling is defective (Fig 2.11E). Thus, ecdysteroid signaling must regulate some other components in this pathway. Overall, we conclude that ecdysteroid signaling in mature follicles regulates follicle rupture by promoting Mmp2 activation (Fig 2.11A).

Discussion

Ecdysteroids regulate ovulation in adult *Drosophila*

For the first time, our study defined the dynamic expression pattern of Shd and EcR isoforms in late oogenesis, and demonstrated the requirement of ecdysteroid signaling in ovulation. Ecdysteroids have also been shown to regulate early germ cell differentiation (Ables and Drummond-Barbosa, 2010; König et al., 2011), egg chamber progression at stage 8-9 (Buszczak et al., 1999; Carney and Bender, 2000; Terashima

et al., 2005), and endocycle-to-gene-amplification switch at stage10B (Sun et al., 2008). This temporal action of ecdysteroids is reminiscent to their roles in regulating multiple larval and pupal transitions (Riddiford et al., 2000; Thummel, 1996). It is, however, unclear whether egg chambers receive pulse-like ecdysteroid titers at these transitions, similar to those received during molting and metamorphosis. Unlike early development, where ecdysone is produced in the ring gland, adult female ecdysteroids are thought to be produced mainly in the ovary, as mRNAs encoding many enzymes in the ecdysone synthesis pathway are detected in follicle cells, nurse cells, or both. Since ecdysteroid secretion is mediated through a regulated vesicular trafficking mechanism (Yamanaka et al., 2015), it is conceivable that follicle cells, nurse cells, or both could produce local pulses of ecdysteroids to promote egg chamber development at each transition during oogenesis. This is consistent with the finding that the ligand sensor for ecdysteroids shows a stage-specific activity including stage 10 and stage 14 (Hackney et al., 2007; Sun et al., 2008). This notion is further supported by this study that upregulation of Shd in mature follicle cells produces 20E to regulate ovulation and a previous study that local ecdysteroids produced in follicle cells regulate border cell migration (Domanitskaya et al., 2014).

Interestingly, we only observed the requirement of Shd in mature follicle cells for normal ovulation, while the involvement of the early enzymes such as Sad, Dib, Phm, and Spo in mature follicle cells are not decisive. It is unlikely that Shd monooxygenates another molecule, instead of E, that regulates ovulation, because 20E can rescue the defect of *shd*-knockdown follicles. We favor the hypothesis that early enzymes for E synthesis function in younger follicle cells (such as stage 13 where Phm protein is highly

expressed; Fig 2.5A-B), which carry E into stage 14, where Shd converts them to 20E. Alternatively, E may be transported into mature follicle cells from outside, possibly from the corpus luteum where Phm is also expressed (Deady et al., 2015). It is also possible that the differential effect of *shd* and earlier enzymes in follicle rupture/ovulation reflects differences in the RNAi knockdown efficiency.

Isoform-specific roles of Ecdysone receptor

EcR is a typical nuclear receptor, belonging to NR1 family (King-Jones and Thummel, 2005). The single *EcR* gene produces three alternatively spliced isoforms with variable N-terminal regions but the same C-terminus, giving them the capacity to bind to the same ligands and DNA sequences. Here, we showed that the regulation of follicle rupture is not only through upregulating 20E production in stage-14 follicles, but also likely by regulating EcR isoform expression. We discovered that both EcR.A and EcR.B1 are downregulated before entering stage 14, unlike a previous report that both isoforms are expressed throughout oogenesis (Carney and Bender, 2000). This downregulation is likely important for follicle rupture as ectopic expression of EcR.A or EcR.B1 interferes with the OA-induced follicle rupture. Our data is consistent with the idea that EcR.B2 is the only EcR isoform functioning in mature follicle cells to mediate the ecdysteroid signaling necessary for ovulation. Thus, upon 20E binding, EcR.B2 may regulate specific arrays of genes for ovulation that cannot be activated or will be interfered by other EcR isoforms. Isoform specific roles of EcR have also been reported in wing margin development and border cell migration (Cherbas et al., 2003); however, follicle rupture is the first biological process showing interference between EcR

isoforms. The specific genes regulated by EcR.B2 and how EcR.B2 fulfills such specific functions are unknown.

Conserved role of steroids in ovulation

The mechanisms underlying ovulation in insects, such as *Drosophila*, were thought to be highly divergent from mammals, in which ovulation is regulated by luteinizing hormone and progesterone, not identified in insects so far. However, our recent work demonstrated the involvement of follicle rupture and matrix metalloproteinase in *Drosophila* ovulation, reminiscent to mammalian ovulation (Curry and Osteen, 2003; Deady and Sun, 2015; Deady et al., 2015). In addition, it seems that adrenergic regulation of calcium signaling is also conserved in regulating ovulation in both *Drosophila* and mammals (Föhr et al., 1993; Breen et al., 2013; Deady and Sun, 2015). The steroid hormone progesterone and progesterone receptor signaling in preovulatory follicles is essential for mammalian ovulation (Lydon et al., 1995). It remained a mystery whether steroid signaling also plays a conserved role in *Drosophila* ovulation. Our study solved this mystery by demonstrating an important role of ecdysteroids, the principle steroid hormones of *Drosophila*, in mature follicle cells for follicle rupture/ovulation. Like preovulatory follicles that only transiently upregulate progesterone receptor in granulosa cells before ovulation (Park and Mayo, 1991), mature follicles of *Drosophila* also adjust their expression of EcR receptors to allow EcR.B2 to remain functioning and promote ovulation. It is interesting that neither progesterone nor ecdysteroid signaling regulates *mmp* expression (Figure 2.11E) (Robker et al., 2000). The downstream targets of ecdysteroid signaling in ovulation are currently unclear; however, it is possible that ecdysteroids may regulate other proteases

enriched in late oogenesis as progesterone signaling does (Robker et al., 2000). Future work on ecdysteroid signaling in *Drosophila* ovulation will thus provide insights into fundamental mechanisms of steroid signaling in ovulation.

Materials and methods

Drosophila genetics

Flies were reared on standard cornmeal-molasses food at 25°C unless otherwise indicated. EcR^{ts} is a trans-heterozygous combination of EcR^{A483T}/EcR^{M554fs} with heterozygous flies as the control (Carney and Bender, 2000). These animals were raised at 22°C and shifted to 29°C upon adult eclosion. *R47A04-Gal4* and *R44E10-Gal4* from the Janelia Gal4 collection (Pfeiffer et al., 2008) were used for misexpressing genes or RNAi in mature follicle cells and their specific expression pattern in female reproductive system were characterized before (Deady and Sun, 2015; Deady et al., 2015). All RNAi-knockdown experiments were performed at 29°C with *UAS-dcr2*. The following RNAi lines were used: *UAS-shd-i1* (V108911), *UAS-shd-i2* (V17203), *UAS-sad-i* (V41269), *UAS-phm-i1* (V108359), *UAS-phm-i2* (V6169), *UAS-dib-i* (V101117), *UAS-spo-i* (V51170) from the Vienna Drosophila Resource Center (VDRC); *UAS-EcR-i* (B9327), *UAS-EcR.A-i* (B9328), *UAS-EcR.B1-i* (B9329) from the Bloomington Drosophila Stock Center (BDSC). The following misexpressing lines were also used: *UAS-EcR.B1*^{DN1} (*UAS-EcR.B1*^{F645A}; B6869), *UAS-EcR.B1*^{DN2} (*UAS-EcR.B1*^{W650A}; B6872), *UAS-EcR.A* (B6470), *UAS-EcR.B1* (B6469), *UAS-EcR.B2* (B6468) from the BDSC; *UAS-shd* (Petryk et al., 2003), *UAS-RFP* (Deady et al., 2015). Control flies were derived from specific Gal4 drivers crossed to *Oregon-R*. The *Mmp2::GFP* fusion allele in

the *Mmp2* endogenous locus was used for detecting Mmp2 protein expression (Deady et al., 2015).

Egg laying and ovulation time

Analysis of egg laying and ovulation time was performed as previously described with minor modifications (Deady and Sun, 2015). In short, 5-6-day-old virgin females were fed with wet yeast 1 day prior to egg laying, and five females and ten *Oregon-R* males were kept in a bottle to lay eggs on grape juice-agar plates for two days at 29°C. After egg laying, ovaries were dissected and mature follicles in these ovaries were quantified. The number of eggs laid on the plates was counted and used to calculate the average time for laying an egg (egg-laying time). The egg-laying time was partitioned into the ovulation time, oviduct time and the uterus time. The partition ratio was determined based on the percentage of females having eggs in the oviducts and uterus at six hours after mating.

***Ex vivo* follicle rupture, *in situ* zymography, qRT-PCR**

Ex vivo follicle rupture and *in situ* zymography were performed as previously described (Deady and Sun, 2015). 5-6-day-old virgin females were used to isolate mature follicles according to fluorescent signals driven by *R47A04-Gal4* or *R44E10-Gal4*. For OA-induced follicle rupture, 20 μM of OA (Sigma) were supplemented in the culture medium and cultured for three hours unless otherwise indicated. For E and 20E cultures, mature follicles were preincubated in the medium with E or 20E (Cayman Chemical) for half an hour before addition of OA. For each culture, about 30 mature follicles were used and the percentage of ruptured follicles (losing more than 80%

follicle-cell covering the oocyte) was calculated. Data were represented as mean ± standard deviation (SD); and Student's T-test was used for statistic analysis.

In situ zymography for detecting gelatinase activity was performed as previously reported (Deady et al., 2015). 25 □g/ml of DQ-gelatin conjugated with fluorescein (Invitrogen) was added into the culture media with or without OA for three hours (or six hours in the case of 20E rescue experiment). After a quick rinse, mature follicles with posterior fluorescent signal were directly counted.

For qRT-PCR, total RNA was extracted from isolated mature follicles with TRIzol (Invitrogen) according to the standard protocol and was used to synthesize cDNA via the SuperScript III First-Strand Synthesis System (Invitrogen) using Oligo(dT) primers. qPCR amplication was performed with iTaq Universal SYBR Green Supermix (Bio-Rad). The primers used were: OambF 5' TGACCAACGATCGGGGTTAT 3' and OambR 5' ATGCGCAATATGAGCTGGGA 3' for detecting *Oamb.K3* isoform expressed in mature follicles (Deady and Sun, 2015), Mmp2F 5' TACTTGTGGCGCATTGGAAC 3' and Mmp2R 5' ATCGATGTGGGTCAAAGTGG 3' for *Mmp2* gene. The *Rps17* is used as an internal control.

Immunostaining and microscopy

Immunostaining was performed following a standard procedure (Sun and Spradling, 2012), including fixation in 4% EM-grade paraformaldehyde for 15 minutes, blocking in PBTG (PBS+ 0.2% Triton+ 0.5% BSA+ 2% normal goat serum), and primary and secondary antibody staining. Rabbit anti-Shd and anti-Phm (1:250; Gifts from Dr. Michael O'Connor), mouse anti-EcR, anti-EcR.B1, anti-EcR.A, and anti-Hnt (1:15; Developmental Study Hybridoma Bank), rabbit anti-RFP (1:4000; MBL International)

and rabbit anti-GFP (Invitrogen) were used as primary antibodies, and Alexa-488 and Alexa-546 goat secondary antibody (1:1000, Invitrogen) were used. Images were acquired using a Leica TCS SP8 confocal microscope or Leica MZ10F fluorescent stereoscope with a sCOMS camera (PCO.Edge), and assembled using Photoshop software (Adobe, Inc.).

Acknowledgements

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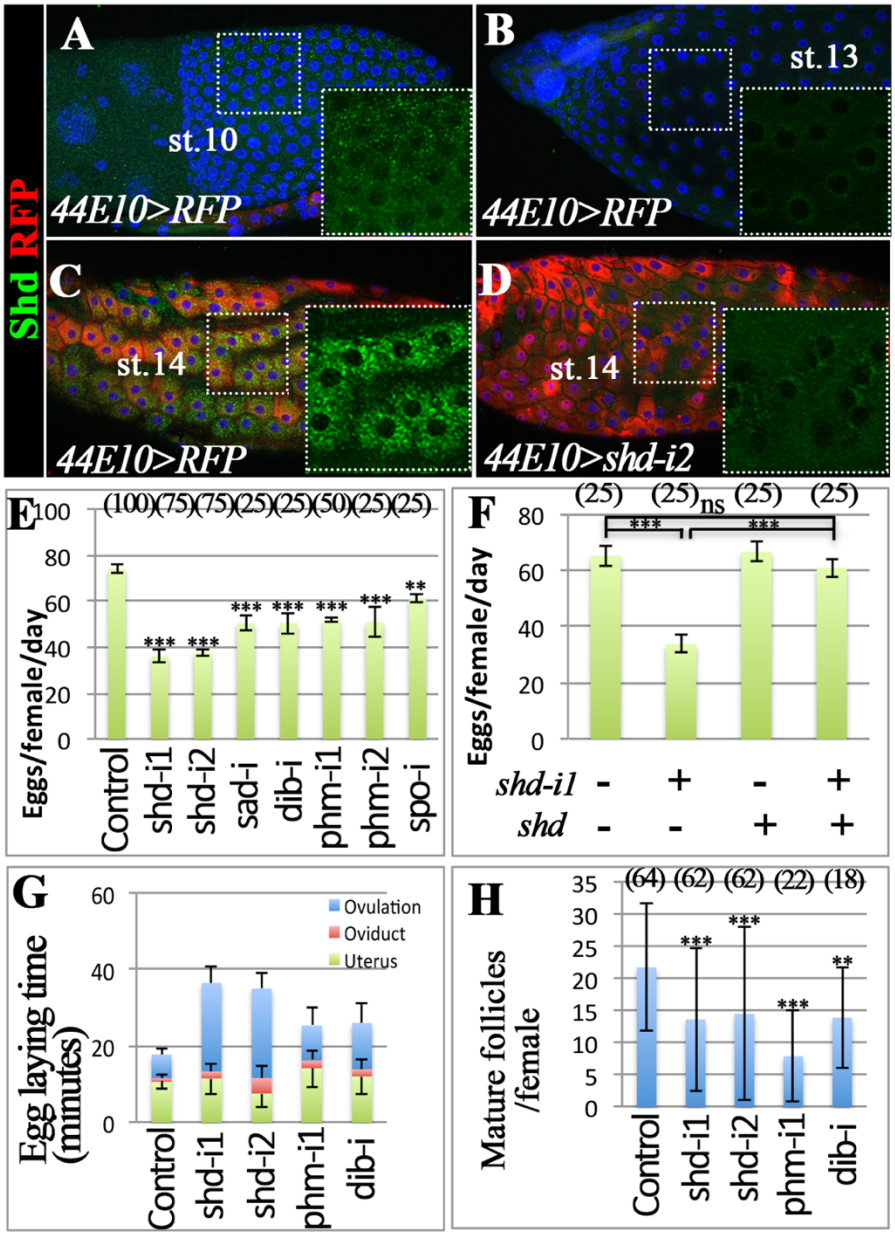

Figure 2.1. Shade is required in mature follicle cells for ovulation.

(A–D) Shd protein expression (green) in egg chambers of control (A–C; R44E10-Gal4 driving UAS-RFP, 44E10 > RFP) and shd-knockdown (D; 44E10 > shd-i2) flies. RFP is in red, and DNA staining with DAPI is shown in blue. The stage of the egg chambers is indicated, and the Insets show the higher magnification of green channel in the outlined area. (E–H) The quantification of egg-laying number (E and F), egg-laying time (G), and mature follicles (H) with specified genotypes. R44E10-Gal4 was used. The number of females analyzed is shown in parentheses, and the error bars are SE for egg-laying number (E and F), 95% confidence interval for egg-laying time (G), and SD for mature follicles (H). ***P < 0.001, **P < 0.01, and *P < 0.05.

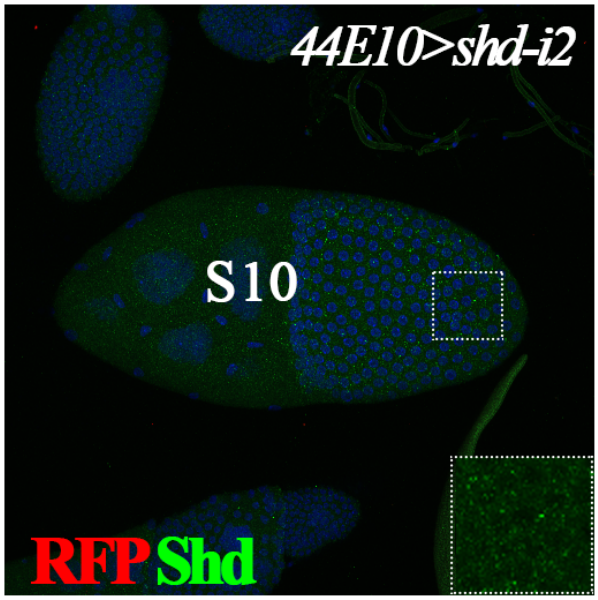

Figure 2.2. Shd expression in stage-10 egg chamber

Expression of Shd (green) is normal in stage-10 follicles from females with *shd* knockdown using *R44E10-Gal4*. *R44E10-Gal4* is not expressed in stage 10 as indicated by *UAS-RFP* expression (in red). DNA staining with DAPI is shown in blue. The insert shows the higher magnification of green channel in the outlined area.

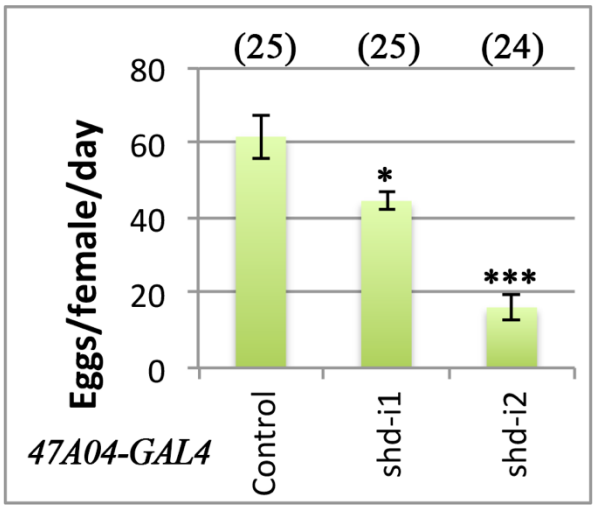

Figure 2.3. Fecundity of females with shd knockdown using R47A04-Gal4

Females with *shd* knockdown lay significantly less eggs than control. Student's T-test was used (*** P<0.001, * P<0.05).

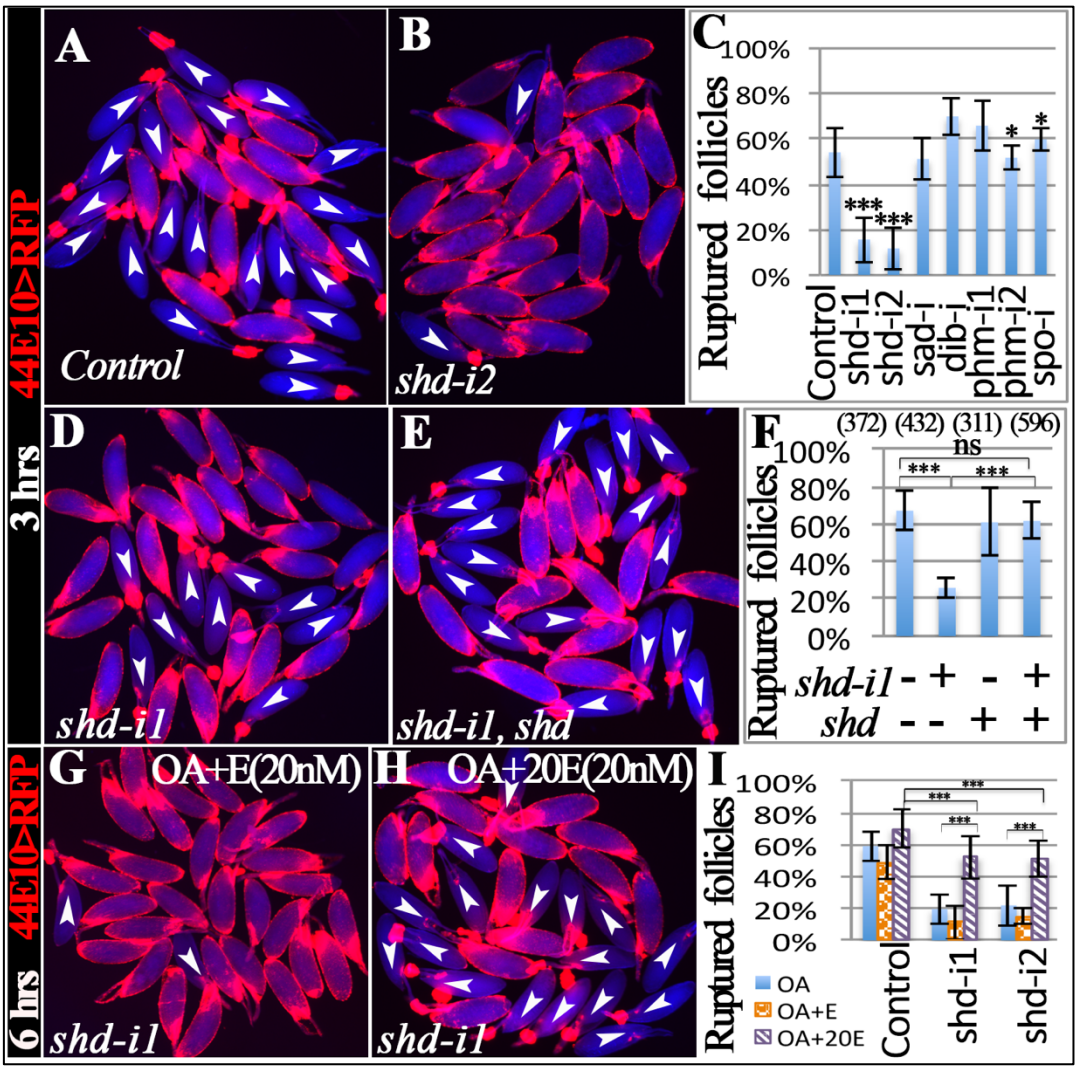

Figure 2.2. Shade is required in mature follicle cells to synthesize 20E for follicle rupture.

For all follicle rupture images here and in subsequent figures, RFP (shown in red) labeling mature follicle cells was overlaid to the bright-field image of follicles (shown in blue). (A-C) *shd* knockdown with *R44E10-Gal4* inhibits follicle rupture. Representative images (A-B) show mature follicles after three-hour culture with OA. The number of mature follicles used in each genotype is 502, 243, 204, 142, 95, 315, 195, and 276. (D-F) *Shd* overexpression rescues the rupture defect of *shd*-knockdown follicles. The number of follicles is listed in the parenthesis. (G-I) 20E but not E can partially rescue rupture defect of *shd*-knockdown follicles after a six-hour culture. The number of follicles used in each condition in (I) is 480, 263, 657, 423, 87, 564, 392, 210, and 563.

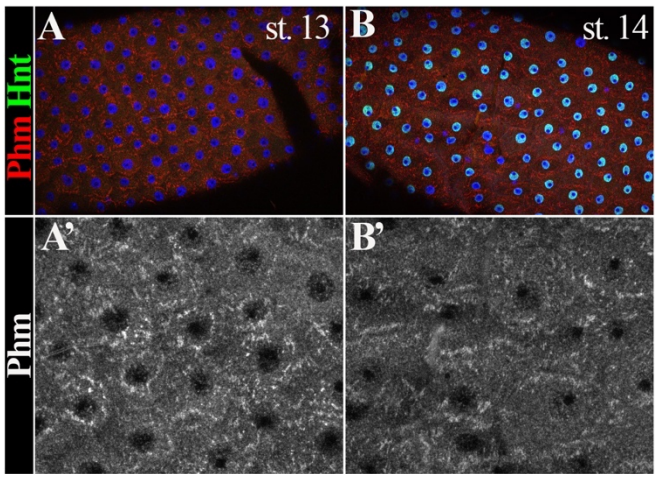

Figure 2.5. Phm expression in stage-13 and stage-14 follicle cells

Endoplasmic reticulum-localized Phm (Red in A-B and white in A'-B') is highly enriched in stage-13 (A-A') and stage-14 (B-B') follicle cells. Transcription factor Hindsight (Hnt; green in A-B) is marking stage-14 follicle cells, but not in stage-13 follicle cells.

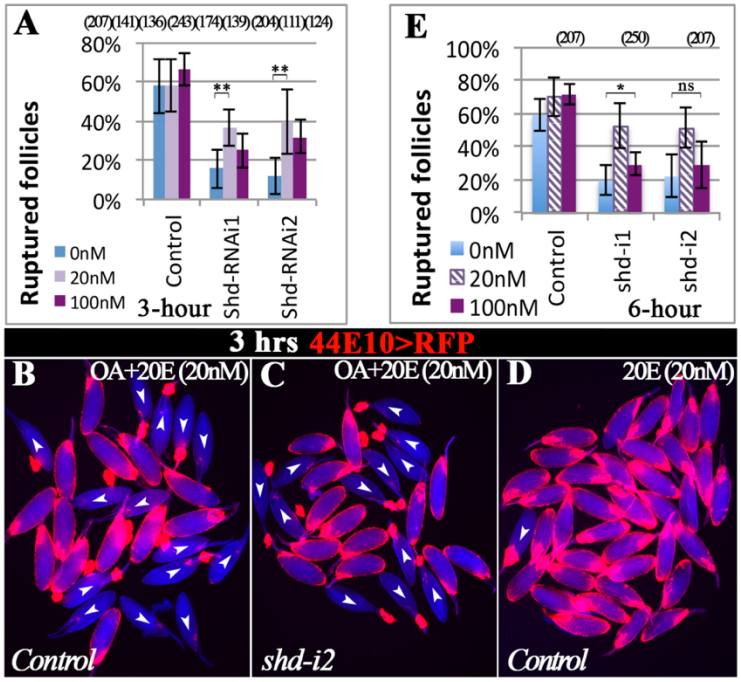

Figure 2.6. Follicle rupture in the presence of OA and 20E

(A) Quantification of follicle rupture with OA and varying concentration of 20E after a 3-hour culture. (B-C) Representative images show mature follicles of control (B) and *shd* knockdown (C) after three-hour culture with OA and 20 nM of 20E.(D) Representative image of control follicles cultured in only 20E (20nM) showing that 20E alone does not induce follicle rupture (3.1%, n=159). (E) Quantification of follicle rupture with OA and varying concentrations of 20E after a 6-hour culture. The groups with 0 and 20 nM 20E are the same as in Fig 2I. Student's T-test was used (** P<0.01; * P<0.05).

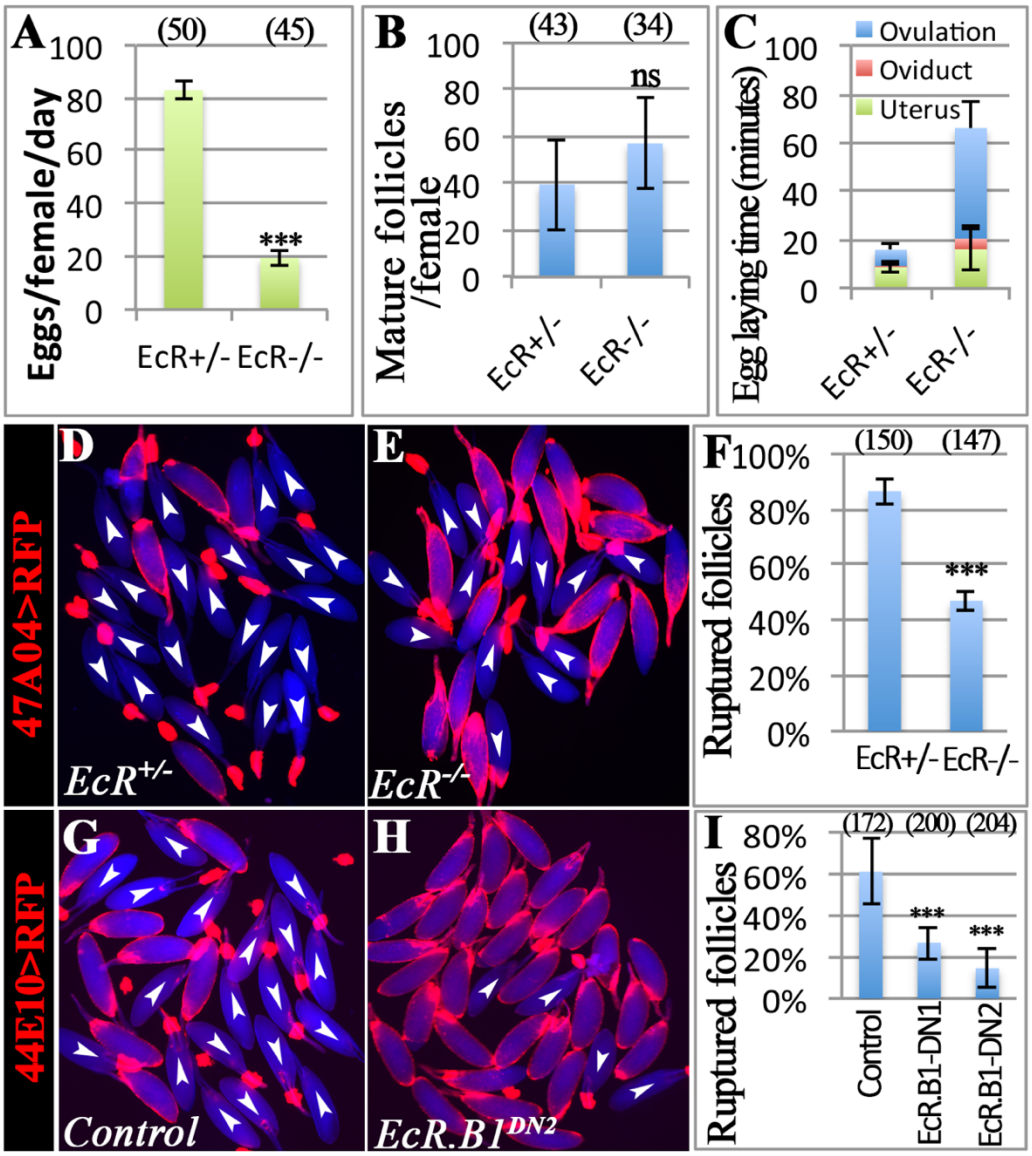

Figure 2.3. EcR is required in mature follicle cells for ovulation.

(A-C) The quantification of egg-laying number (A), mature follicles (B), and egg-laying time (C) in heterozygous control or *EcR*^{ts} females. (D-F) *EcR*^{ts} mature follicles are defective in OA-induced follicle rupture. Mature follicles were marked by *R47A04>RFP*. (G-I) *EcR*^{DN} overexpression using *R44E10-Gal4* inhibits follicle rupture. The number of females or mature follicles is listed in the parenthesis.

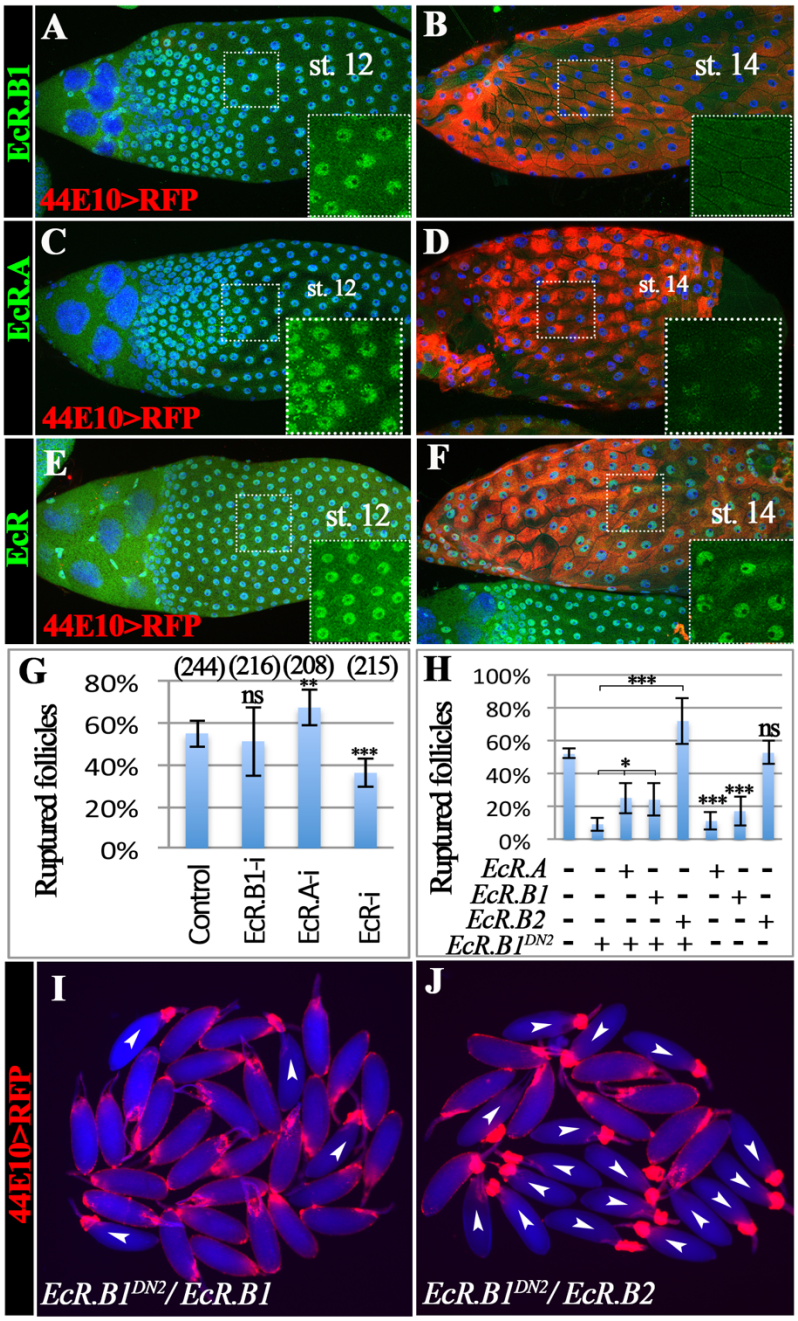

Figure 2.4. EcR.B2 functions in mature follicle cells for follicle rupture.

(A-B) EcR.B1 protein expression (green) in late oogenesis. *RFP* expression driving by *R44E10-Gal4* (*44E10>RFP*) is shown in red, marking stage-14 egg chambers. DAPI is shown in blue. (C-D) EcR.A protein expression in late oogenesis. (E-F) EcR protein expression (including all three isoforms in green) in late oogenesis. (G) Quantification of OA-induced follicle rupture when *EcR* isoforms were knocked down with *R44E10-Gal4*. (H-J) Quantification of OA-induced rupture in follicles with misexpressing *EcR* isoforms and/or EcR^{DN}. Representative images show mature follicles *with R44E10-Gal4 driving* $EcR.B1^{DN2}$ */EcR.B1* (I) and $EcR.B1^{DN2}$ */EcR.B2* (J) after the three-hour culture with OA. The number of follicles used in each condition is 172, 99, 265, 214, 230, 118, 187, and 183.

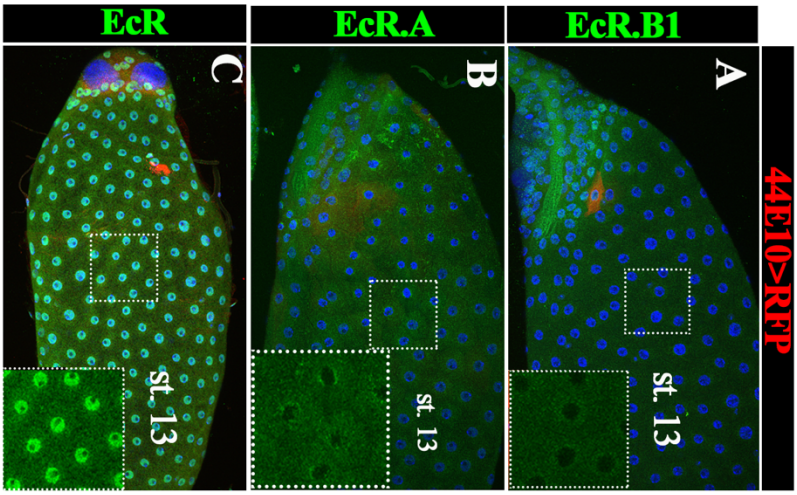

Figure 2.9. EcR isoform expression in stage-13 egg chambers

(A-B) EcR.B1(A) and EcR.A(B) protein (green) are not expressed in stage-13 egg chambers. (C) EcR protein expression (including all three isoforms in green) is expressed in stage-13 egg chambers. *R44E10-Gal4* is not expressed in stage 13 as indicated by *UAS-RFP* expression (in red). DNA staining with DAPI is shown in blue. The insert shows the higher magnification of green channel in the outlined area.

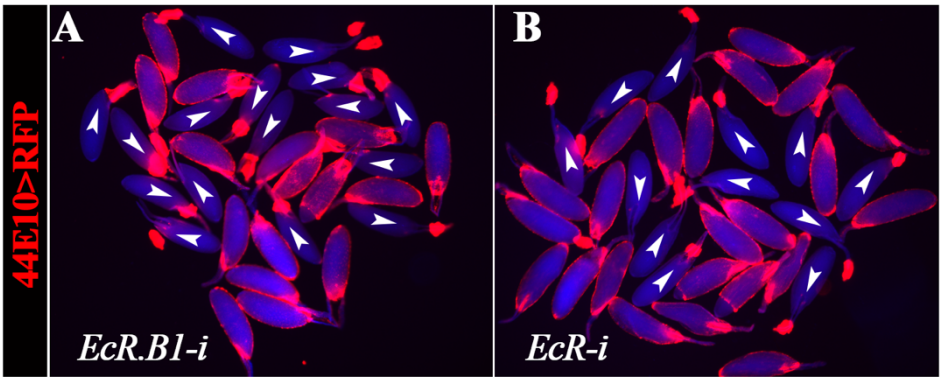

Figure 2.10. Knockdown of EcR-i causes follicle rupture defect.

(A-B) The representative image shows follicles after the three-hour OA stimulation. Mature follicles were expressing RNAi against either EcR.B1 (A) or EcR common region (B) in their follicle cells using *R44E10-Gal4*.

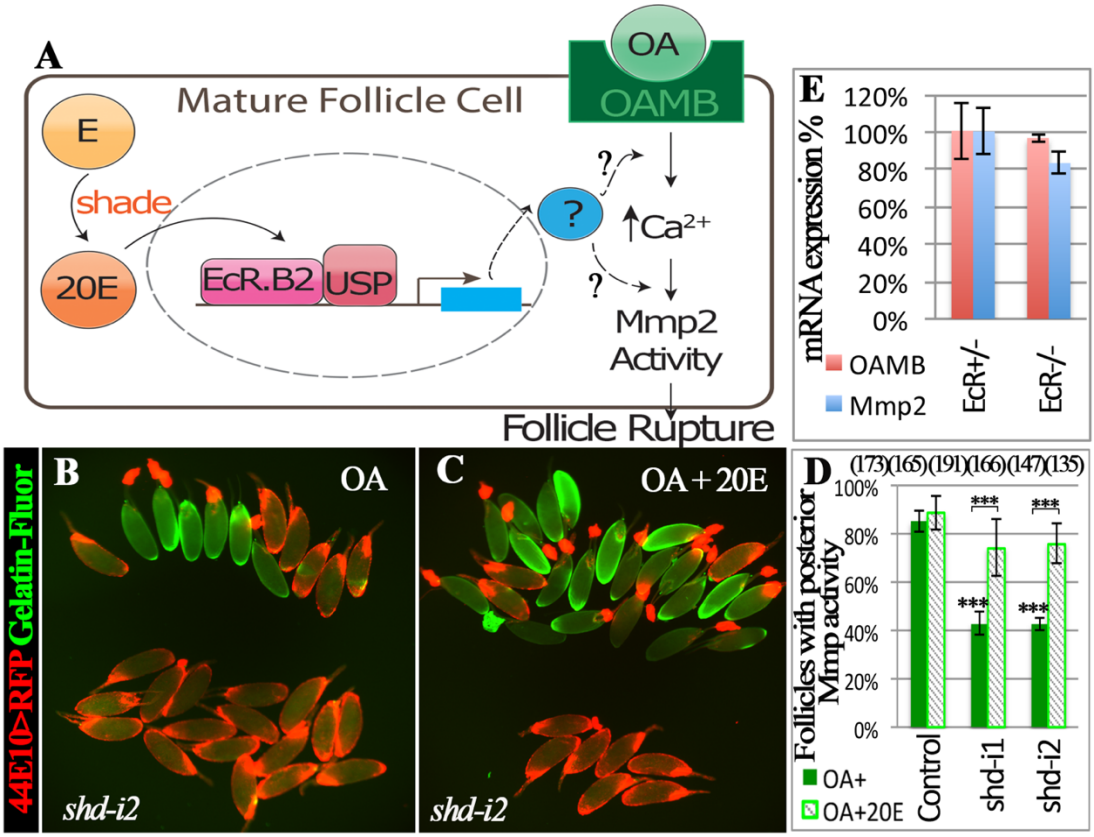

Figure 2.5. Ecdysteroid signaling regulates OA-induced Mmp2 activation.

(A) A schematic showing the model of ecdysteroid signaling in the mature follicle cell to induce follicle rupture. (B-D) *In situ* zymography shows the reduction of gelatinase activity (green in B-C) in *shd*-knockdown follicles (B), which can be rescued by 20 nM of 20E (C). Follicles with posterior gelatinase activity after a six-hour stimulation were separated toward upper region in B-C and quantified in D. (E) Quantification of *Oamb* and *mmp2* mRNA in mature follicles of control and EcR^{ts} females with qRT-PCR.

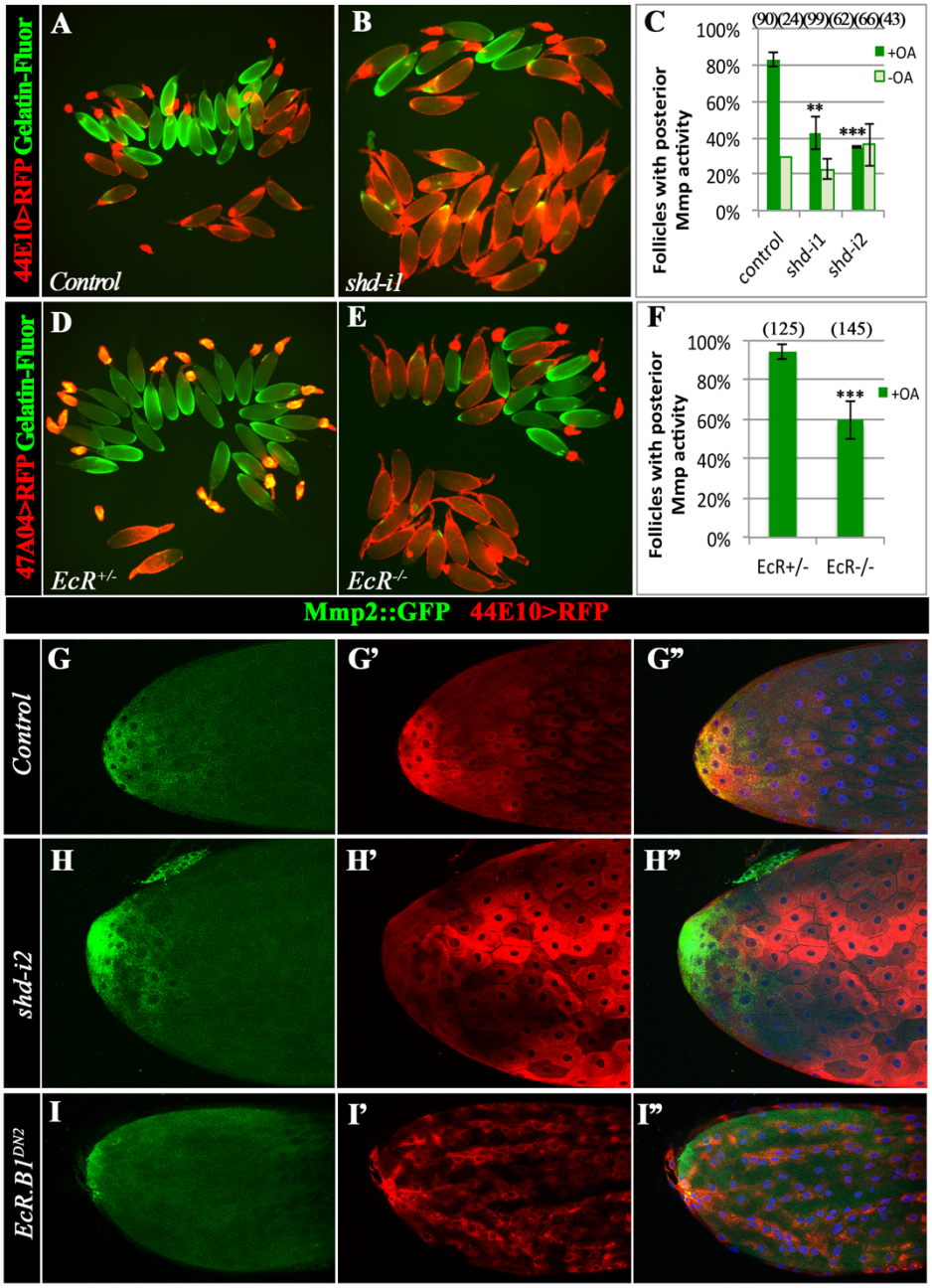

Figure 2.6. Ecdysteroid signaling affects Mmp2 activation but not protein expression.

(A-C) *In situ* zymography shows the reduction of gelatinase activity (green in A-B) in *shd*-knockdown follicles after a three-hour OA stimulation. Follicles with posterior gelatinase activity (green in A-B) are separated toward upper panel in A-B, and the quantification is shown in C. The number of follicles analyzed is in the parenthesis. (D-F) *In situ* zymography shows the reduction of gelatinase activity in EcR^{ts} mutant follicles after a three-hour OA stimulation. (G-I) Mmp2::GFP expression (green in G-I and G"-I") is detected in posterior follicle cells of control (G-G"), *shd*-knockdown (H-H"), and EcR^{DN2} (I-I") egg chambers.

Genotype	Egg laying in 2 days#		Egg distribution in 6h			Egg laying time (min)			
	N	Eggs/ female/ day	N	Uterus with egg (%)	Oviduct with egg (%)	Total time	Ovulation time	Oviduct time	Uterus time
UAS-dcr2/+; 44E10-Gal4/+ (Ore-R)	100	74.2 ± 0.9	90	60.0 ± 10.1	5.6 ± 4.7	17.8 ± 0.2	6.1 ± 1.7	1.0 ± 0.8	10.7 ± 1.8
UAS-dcr2/shd-i1; 44E10-Gal4/+	75	36.2 ± 1.4***	60	31.7 ± 11.8	5.0 ± 5.5	36.5 ± 1.4	23.1 ± 4.5***	1.8 ± 2.0	11.6 ± 4.3
UAS-dcr2/+; 44E10-Gal4/shd-i2	75	37.6 ± 0.6***	60	21.7 ± 10.4	11.7 ± 8.1	35.1 ± 0.6	23.4 ± 4.2***	4.1 ± 2.9	7.6 ± 3.7
UAS-dcr2/phm-i; 44E10-Gal4/+	50	51.9 ± 0.6***	25	56.0 ± 19.5	8.0 ± 10.6	25.4 ± 0.3	9.2 ± 4.8	2.0 ± 2.7	14.2 ± 4.9
UAS-dcr2/dib-i; 44E10-Gal4/+	25	50.6 ± 4.1***	28	46.4 ± 18.5	7.1 ± 9.5	26.1 ± 2.1	12.1 ±4.9	1.9 ± 2.5	12.1 ± 4.9
EcR^{A483T}/SM6b	50	82.9 ± 2.1	62	54.8 ± 12.4	3.2 ± 4.4	15.9 ± 0.4	6.7 ± 2.0	0.5 ± 0.7	8.7 ± 2.0
EcR^{A483T}/EcR^{M554fs}	45	19.6 ± 2.1***	46	23.9 ± 12.3	6.5 ± 7.1	67.5 ± 7.4	45.5 ± 10.4***	4.4 ± 4.8	16.1 ± 8.5

Table 1 The effect of ecdysteroid signaling on egg laying, egg distribution in the reproductive tract, and egg-laying time.

one day =22h at 29 °C

* P<0.05, ** P<0.01, *** P<0.001

All data are mean ± 95% confidence interval. Student's T-test was used for egg laying, and Z-Score test was used for egg laying time assuming normal distribution.

Chapter 3 : Downregulation of Homeodomain Protein Cut is Essential for Drosophila Follicle Maturation and Ovulation

Data presented in this chapter is published in the following paper:

Knapp, E.M., Li, W., and Sun, J. (2019). Downregulation of homeodomain protein Cut is essential for Drosophila follicle maturation and ovulation. Development 146, dev179002.

Introduction

Oogenesis is a process of generating mature follicles that are destined to release functional oocytes for fertilization. Although anatomical structure of ovaries is hugely different in diverse species, many molecular/cellular principles governing oogenesis are conserved across different species (Matova and Cooley, 2001). For example, oocyte differentiation from insects to mammals involves incomplete cytokinesis and the receiving of organelles and cytoplasm from sister germ cells (Lei and Spradling, 2016; Matova and Cooley, 2001). Oocyte development is fulfilled in a follicle unit, which consists of somatic follicle cells that encase the oocyte. Proper proliferation and differentiation of somatic follicle cells are essential to nourish oocyte development and ultimately release the functional oocytes through ovulation (Khristi et al., 2018; Robker et al., 2018); however, the molecular mechanisms regulating somatic follicle cell differentiation and follicle maturation in the final pre-ovulatory stages are poorly understood.

The somatic follicle cells encapsulating oocytes in *Drosophila* ovaries provide an exceptional model to study the progressive regulation of cell proliferation, differentiation, and maturation. Each ovary in *Drosophila* consists of ~16 ovarioles, which include a string of developing follicles (egg chambers) wrapped by the circular muscle sheath (Spradling, 1993). At the anterior tip of the ovariole (also named germarium), germline stem cells proliferate to give rise to a 16-cell germline cyst, which will be wrapped by a layer of somatic follicle cells derived from follicle stem cells to form a stage-1 egg chamber at the end of the germarium (Margolis and Spradling, 1995). Each egg chamber develops through 14 distinct stages to become a mature follicle (Spradling, 1993). Somatic follicle cells undergo three separate cell cycle phases: follicle cells are in the mitotic cycle to produce ~855 follicle cells to cover the growing germ cells from stages 1-6; they enter three rounds of endocycles to increase DNA content without cell division from stages 7-10A; and they undergo gene amplification to increase the copy number of chorion genes from stages10B-13 in order to produce the eggshell (Calvi et al., 1998; Edgar and Orr-Weaver, 2001; Klusza and Deng, 2011; White et al., 2009). Homeodomain transcription factor Cut is expressed in stage 1-6 follicle cells and required for follicle cell proliferation (Sun and Deng, 2005). The transition from mitotic cycle to endocycle (M/E transition) is induced by Notch signaling, which activates the zinc-finger transcription factor Hindsight (Hnt) to downregulate Cut and inhibit Hedgehog signaling (Deng et al., 2001; St. Johnston, 2001; Sun and Deng, 2007). In the last decade, multiple factors were discovered to regulate Notch signaling and the M/E transition (Domanitskaya and Schüpbach, 2012; Fic et al., 2019; Jia et al., 2015; Jouandin et al., 2014; Lee and Spradling, 2014; Lo et al., 2019; Poulton et al., 2011;

Starble and Pokrywka, 2018; Vaccari et al., 2010). At the endocycle/gene amplification (E/A) transition, downregulation of Notch signaling permits the activation of ecdysteroid signaling, which upregulates another zinc-finger transcription factor Tramtrack-69 (Ttk69) at stage 10B (Sun et al., 2008). Regulated by microRNA-7, Ttk69 upregulates Cut expression, suppresses Hnt expression, and promotes the chorion gene amplification at stage 10B (Sun et al., 2008; Huang et al., 2013). In addition, ecdysteroid signaling also regulates proper follicle cell migration (Hackney et al., 2007), apical microvilli morphogenesis partly through Ttk69 (Romani et al., 2015), and microRNA-318 expression, which also promote the E/A switch independent of Ttk69 (Ge et al., 2015).

Several populations of somatic follicle cells are established at stage 10B (Fig. 1A), and they go through complex morphogenetic events during stages 10B-14 to synthesize the sophisticated eggshell capable of protecting the developing embryo (Cavaliere et al., 2008; Duhart et al., 2017; Osterfield et al., 2017; Waring, 2000). In addition to regulate chorion gene amplification, Ttk69 plays essential roles for the formation of dorsal appendages (French et al., 2003; Peters et al., 2013). Upregulated Cut in centripital follicle cells counteracts C/EBP transcription factor Slow border cells to regulate centripital follicle cell migration (Levine et al., 2010). In addition, multiple eggshell products have been identified, and temporal gene expression patterns in stage10B-14 follicles have been characterized (Fakhouri et al., 2006; Niepielko et al., 2014; Pyrowolakis et al., 2017; Tootle et al., 2011). Despite extensive work in eggshell formation, it still remains unknown how somatic follicle cells transition into a final maturation status at stage 14 to be ready for ovulation.

We recently characterized the cellular process of ovulation in *Drosophila* and identified multiple factors functioning in stage-14 follicle cells for ovulation. Like mammals, *Drosophila* ovulation involves an initial step of follicle wall breakdown by trimming posterior follicle cells, a process dependent on matrix metalloproteinase 2 (Mmp2) (Deady et al., 2015). The exposed oocyte then ruptures into the oviduct, while the rest of the follicle cells compress and remain in the ovary. This process depends on NADPH oxidase (Nox), which produces the signaling molecule hydrogen peroxide in the presence of superoxide dismutase 3 (SOD3) (Li et al., 2018). Both Mmp2 and Nox are activated by elevated intracellular calcium, which is induced by the octopamine (OA) binding to its receptor octopamine receptor in mushroom body (Oamb) in stage-14 follicle cells (Li et al., 2018; Deady and Sun, 2015).

We also discovered that Hnt is re-upregulated in stage-14 follicle cells and is essential for Mmp2 expression in posterior follicle cells at stage 14B and Oamb expression in all follicle cells at stage 14C, a final maturation stage (Deady et al., 2017). Furthermore, the enzyme Shade (Shd) required for 20-hydroxyedcysone (20E) synthesis is also upregulated in stage-14 follicle cells, while two of its receptors (EcR.A and EcR.B1) are downregulated, which leads to specific ecdysteroid signaling via EcR.B2 in stage-14 follicle cells for follicle rupture and ovulation (Knapp and Sun, 2017). Thus, it seems there could be another critical follicle cell transition from stage 13 to stage 14 to allow follicle cells to mature into a fully competent preovulatory state. The question still remains as to what actually regulates this transition of follicle cells into an ovulatory competent state.

In this study, we characterize the follicle cell transition at stage 13/14. We report that Cut, homologue of human CCAAT displacement protein (Ellis J Neufield et al., 1992), is downregulated at stage 13/14 transition. Its downregulation is crucial for this transition as extended expression of Cut into stage-14 follicle cells leads to ovulation defects and suppression of Hnt upregulation, a key regulator in follicle rupture. Furthermore, continuation of Cut expression in stage-14 follicle cells also promotes Ttk69 expression, whose downregulation in stage-14 follicle cells is also crucial for full activation of Oamb signaling. Our findings elucidate a maturation pathway involving downregulation of Cut/Ttk69 and upregulation of Hnt at the stage13/14 transition that is necessary for follicle cells to transition into stage 14 and thus gain ovulatory competency.

Results

The expression of Cut and Ttk69 inversely correlates with Hnt in late oogenesis

Our recent work demonstrated the critical role of Hnt upregulation in stage-14 follicle cells for ovulation; however, little is known about the mechanism of Hnt upregulation and follicle maturation. To identify potential transcription factors that regulate the follicle cell transition into stage-14 preovulatory stage, we characterized the expression of several transcription factors in late oogenesis. Our experiments confirmed the previous finding that Hnt is downregulated in mainbody follicle cells after stage 10B, through stages 11-13, and re-upregulated at stage 14 (Fig. 3.1B-E)(Deady et al., 2015; Sun et al., 2008). Interestingly, we observed that Cut and Ttk69 showed an inverse expression pattern to Hnt from stage 10B to stage 14. After upregulated at stage 10B,

Cut expression exhibited a steady decrease in mainbody follicle cells and was undetectable by stage 14, when Hnt is upregulated (Fig. 3.1F-I). Ttk69 showed a similar expression pattern as Cut and was also undetectable in stage-14 mainbody follicle cells (Fig. 3.1J-M).

The antagonistic relationship between Hnt and Cut at the M/E switch in stage-6/7 follicle cells led us to hypothesize that Hnt upregulation in stage-14 follicle cells downregulates Cut expression. To test this hypothesis, we knocked down *hnt* in stage-14 follicle cells with *44E10-Gal4* driver (Deady and Sun, 2015) and examined the Cut expression. Cut expression is undetected in stage-14 follicle cells (data not shown). Since *44E10-Gal4* is not efficient to knock down *hnt* in early stage 14 (stage 14A; Deady et al., 2017), we generated an alternative Gal4 line (*CG13083-Gal4*), which is expressed in stage-13 follicle cells (Fig. 3.2A). *hnt* was completely knocked down in stage-14 follicle cells with *CG13083-Gal4*; however, Cut was still undetectable in these mainbody follicle cells, nor was Ttk69 (Fig. 3.2B-E). Therefore, upregulation of Hnt is not the cause of downregulation of Cut and Ttk69 in stage-14 follicle cells.

Downregulation of Cut in stage-14 follicle cells is required for ovulation/follicle rupture

The finding that Hnt does not downregulate Cut and Ttk69 expression in stage-14 follicle cells led us to question whether their downregulation is important for follicle maturation. To address this question, we extended Cut expression in stage-14 follicle cells with *44E10-Gal4* driver (Fig. 3.3A-B). Females bearing such genetic manipulation laid significantly fewer eggs compared to the control females (Fig. 3.3C). In addition, ovaries from these females retained significantly more mature follicles after egg laying

(Fig. 3.3D). No morphological defects were observed in mature follicles. These results suggest that ectopic Cut expression in stage-14 follicle cells leads to an ovulation defect.

Drosophila ovulation involves a proteolytic degradation of posterior follicle cells and follicle rupture to release the oocyte into the oviduct (Deady and Sun, 2015; Deady et al., 2015). This follicle rupture process is stimulated by octopaminergic signaling in stage-14 follicle cells and can be recapitulated in our *ex vivo* culture system (Deady and Sun, 2015; Knapp et al., 2018). To test whether ectopic Cut in stage-14 follicle cells disrupts follicle rupture, we isolated mature follicles and stimulated with OA in our *ex vivo* culture system. Consistent with the ovulation defect observed *in vivo*, *cut*-overexpressing follicles showed less than 3% follicle rupture in comparison to ~50% follicle rupture in control follicles (Fig. 3.3E-G). Together, these results suggest that downregulation of Cut in stage-14 follicle cells is essential for proper follicle rupture and ovulation.

Ectopic Cut blocks Mmp activity and ROS production in mature follicle cells

To determine why *cut*-overexpressing follicles are defective in OA-induced follicle rupture, we first investigated whether these follicles can respond to ionomycin stimulation, which bypasses OA/Oamb to induce calcium influx and follicle rupture (Deady and Sun, 2015). Control follicles demonstrated a robust rupture of 95% in response to ionomycin; however mature follicles with ectopic Cut showed less than 6% follicle rupture, indicating that these follicles are defective in ovulatory signaling downstream of calcium influx (Fig. 3.4A-C).

Our recent work showed that OA-induced calcium elevation in stage-14 follicle cells leads to Mmp2 activation and ROS production, both of which are required for follicle rupture (Deady and Sun, 2015; Li et al., 2018) . To determine if ectopic Cut blocks Mmp2 activation in mature follicle cells, we performed *in situ* zymography in control and *cut*-overexpressing follicles to examine OA-induced Mmp2 activation. Consistent with previous finding, approximately 63.8% of control follicles exhibited posterior gelatinase activity when stimulated with OA (Fig. 3.4D-E; (Deady and Sun, 2015). In contrast, only ~15.5% of *cut*-overexpressing follicles had posterior gelatinase activity (Fig. 3.4D and 3F), indicating that *cut*-overexpressing follicles are defective in OA-induced Mmp2 activation. Next, we examined OA-induced superoxide production in control and *cut*-overexpressing follicles. To do this, we isolated stage-14 follicles and performed a luminescence assay utilizing the dye L-012 to detect the levels of superoxide. Our results showed that OA is able to induce a robust increase in superoxide production in control follicles, consistent with previous results (Li et al., 2018). However, *cut*-overexpressing follicles showed minimal, if any, superoxide production after OA stimulation (Fig. 3.4G). Altogether, these results demonstrated that ectopic expression of Cut is sufficient to inhibit follicle rupture through disrupting both Mmp2 activation and ROS generation.

Ectopic Cut prevents the upregulation of Oamb, Mmp2, and Hnt in stage-14 follicle cells

To find out how ectopic Cut inhibits Mmp2 activation and ROS production, we first examined the expression of Mmp2 and Nox, the key enzyme in ROS production, in stage-14 follicles. Using a *Mmp2::GFP* fusion gene, we found that Mmp2 was detected

in posterior follicle cells of control, but not in *cut*-overexpressing stage-14 follicles (Fig. 3.5A-B). To confirm this result, we also analyzed the *Mmp2* mRNA in stage-14 follicles. To our surprise, *cut*-overexpressing follicles only showed minimal decrease in *Mmp2* mRNA level (Fig. 3.5C). However, closer examination of *Mmp2::GFP* expression elucidated that ectopic Cut only downregulated *Mmp2* mRNA in posterior follicle cells, but not in anterior follicle cells (Fig. 3.6A-B). This is further supported by qRT-PCR analysis with either posterior or anterior halves of mature follicles. *Mmp2* mRNA is significantly reduced in posterior halves of *cut*-overexpressing follicles but not in anterior halves of those follicles (Fig. 3.6C-D). Therefore, ectopic Cut prevents the upregulation of *Mmp2* mRNA and protein in posterior follicle cells. It also suggests that regulation of Mmp2 expression in posterior and anterior follicle cells utilizes different mechanisms. Overall, these results indicate that downregulation of Cut is required for the proper upregulation of Mmp2 in posterior follicle cells.

Next, we measured the expression of *Nox* in control and *cut*-overexpressing follicles. We did not observe any significant difference in *Nox* mRNA levels (Fig. 3.5C); however, we did see a significant decrease in the level of *Oamb* mRNA in *cut*-overexpressing follicles compared to controls (Fig. 3.5C), indicating that ectopic Cut inhibits *Oamb* expression. To support this conclusion, we analyzed the expression of *Oamb-RFP*, a reporter with *Oamb* enhancer region fused with *RFP* gene, in control and *cut*-overexpressing follicles. *Oamb-RFP* is uniformly expressed in stage-14 follicle cells of control egg chambers (Fig. 3.5D). In contrast, *Oamb-RFP* expression is considerably reduced in *cut*-overexpressing follicle cells (Fig. 3.5E). Together, these data suggest

that ectopic Cut inhibits Oamb expression in all stage-14 follicle cells and Mmp2 expression in posterior follicle cells.

Since both Oamb and Mmp2 are downstream targets of Hnt (Deady et al., 2017), we therefore hypothesize that ectopic Cut inhibits Hnt expression. Consistent with this hypothesis, we observed that Hnt is severely disrupted in stage-14 follicle cells with ectopic Cut expression (Fig. 3.5F-G). Altogether, our data suggest that downregulation of Cut in stage-14 follicle cells is essential for upregulation of Hnt, which promotes Oamb and Mmp2 expression.

Cut regulates other factors for ovulation in addition to Hnt

To determine whether loss of Hnt in *cut*-overexpressing follicles is responsible for the loss of Mmp2, Oamb, and ovulatory competency, we set to rescue the ovulation defect of *cut*-overexpressing females with ectopic Hnt. Antibody staining confirmed that both Cut and Hnt were robustly expressed in stage-14 follicle cells when driven by *44E10-Gal4* driver (Fig. 3.7A-B). Females with ectopic Cut and Hnt expression in stage-14 follicle cells (*cut/hnt*-overexpressing females) only showed minimal increase of the egg-laying capacity in comparison to females with ectopic Cut alone and were significantly different from control females (Fig. 3.7C). In addition, *cut/hnt*-overexpressing females still showed egg retention after a two-day egg-laying experiment (Fig. 3.7D), and follicles from these females did not respond to OA or ionomycin stimulation (Fig. 3.7E-F). Furthermore, *cut/hnt*-overexpressing follicles were still defective in OA-induced ROS production (Fig. 3.7G).

Interestingly, when examining Mmp2 activation upon OA stimulation, we observed that significantly more *cut/hnt*-overexpressing follicles showed posterior gelatinase activity than *cut*-overexpressing follicles; however, the rate of gelatinase activity in *cut/hnt*-overexpressing follicles was still significantly lower than control follicles (Fig. 3.7H). This result suggests that overexpression of Hnt partially restores the ability of the *cut*-overexpressing follicles in OA-induced Mmp2 activation. Consistent with these findings, we also observed partial restoration of Mmp2 and Oamb expression in *cut/hnt*-overexpressing follicles. More than 50% of *cut/hnt*-overexpressing follicles exhibited moderate or high-level of Mmp2::GFP expression in their posterior follicle cells, while only ~27% of *cut*-overexpressing follicles had moderate or high-levels of Mmp2::GFP expression (Fig. 3.7I-K). In addition, qRT-PCR analysis showed that *cut/hnt*-overexpressing mature follicles exhibited increased levels of *Oamb* mRNA, compared to *cut*-overexpressing follicles (Fig. 3.7L). Altogether, these results suggest that loss of Hnt in *cut*-overexpressing follicles is partially responsible for the loss of Oamb and Mmp2 expression, consistent with Hnt's role in regulating these genes. In addition, these results suggest that Cut regulates additional factors aside from Hnt to prevent full expression of Oamb and Mmp2, as well as ROS production, which leads to defective follicle rupture/ovulation.

Ectopic Cut promotes Ttk69 expression in stage-14 follicle cells

To determine what other factors could be regulated by Cut expression in stage-14 follicle cells, we analyzed the expression of genes that show differential expression at stage-13/14 transition. Both EcR.A and EcR.B1, two isoforms of ecdysone receptor,

are downregulated in stage-14 follicle cells, while the 20E-synthesizing enzyme Shd is robustly upregulated in stage-14 follicle cells (Knapp and Sun, 2017). All of these proteins still exhibited normal expression patterns in *cut*-overexpressing follicle cells (Fig. 3.8A-F), indicating that ectopic Cut unlikely targets ecdysone signaling to disrupt follicle rupture and ovulation. Since Ttk69 is also downregulated in stage-14 follicle cells (Fig. 3.1M), we then examined Ttk69 expression in *cut*-overexpressing follicles. Interestingly, Ttk69 was still detected in the nuclei of stage-14 follicle cells with Cut overexpression but was undetectable in the nuclei of control follicle cells (Fig. 3.9A-B). These findings suggest that ectopic Cut is sufficient to promote Ttk69 expression in stage-14 follicle cells.

Ectopic Ttk69 in stage-14 follicle cells disrupts ovulation/follicle rupture

To determine whether the expression of Ttk69 in stage-14 follicle cells leads to an ovulation defect, we directly overexpressed *ttk69* in stage-14 follicle cells using *44E10-Gal4* driver. Unfortunately, females with such genetic manipulation are lethal, likely due to *44E10-Gal4* driving *ttk69* expression in developmental stages. We thus included the *tubGal80^{ts}* to bypass the developmental defect by culturing the animals in 18 °C and shifted the newly eclosed females to restrictive temperature 29 °C. Females with such genetic manipulations were viable, and Ttk69 was indeed overexpressed in stage-14 follicle cells (Fig. 3.9C). Consistent with our hypothesis, *ttk69*-overexpressing females had a severely decreased egg-laying rate and showed a significant retention of mature follicles after an egg-laying experiment (Fig. 3.9D-E). In addition, mature follicles with Ttk69 overexpression were defective in OA-induced follicle rupture and

ionomycin-induced follicle rupture (Fig. 3.9F-G). Furthermore, these follicles were also defective in Mmp2 activation and ROS production (Fig. 3.9H-I). All these defects were similar to those in *cut*-overexpressing follicles.

Next, we investigated whether ectopic Ttk69 affects Oamb, Mmp2, and Hnt expression. Similar to *cut*-overexpressing follicles, *ttk69*-overexpressing follicles showed normal expression of *Nox*, but significant reduction in levels of *Oamb* and *Mmp2* mRNA (Fig. 3.9J). The reduction of Mmp2 mRNA is also limited to posterior halves of the follicles (Fig. 3.6C). Unfortunately, due to the complication of the genetic crosses, we were unable to examine the Mmp2::GFP expression directly in *ttk69*-overexpressing follicle cells. Additionally, we found that ectopic Ttk69 did not affect the expression of Hnt in stage-14 follicle cells (Fig. 3.9K-L), nor did it extend Cut expression in stage-14 follicle cells (Fig. 3.104). Taken together, these results suggest that ectopic Ttk69 in stage-14 follicle cells is able to disrupt follicle rupture by preventing Oamb and Mmp2 expression independent of Hnt and Cut.

Both Ttk69 downregulation and Hnt upregulation are required for the full activation of Mmp2 during ovulation

Our study showed that Ttk69 is upregulated in *cut*-overexpressing follicles and that ectopic Ttk69 mimics *cut* overexpression independent of Hnt. In addition, we also found that Hnt did not block Ttk69 expression in stage-14 follicle cells as Ttk69 was still detected in *cut/hnt*-overexpressing follicle cells (Fig. 3.11A). Therefore, we hypothesized that Ttk69 is the additional factor, aside from Hnt, to regulate follicle rupture in *cut*-overexpressing follicles. To test this hypothesis, we aimed to rescue the ovulation defect of *cut*-overexpressing females by simultaneously overexpressing *hnt*

and knocking down *ttk* with RNA interference (RNAi; for simplicity, we named these females as *cut/hnt/ttk*^{RNAi}-overexpressing females). Antibody staining confirmed that Ttk69 was indeed knocked down in stage-14 follicle cells of *cut/hnt/ttk*^{RNAi}-overexpressing females (Fig. 3.11B). To our surprise, *cut/hnt/ttk*^{RNAi}-overexpressing females still showed an egg-laying defect and follicle retention (Fig. 3.11C-D), indicating an ovulation defect. Mature follicles from these females did not rupture in response to OA stimulation (Fig. 3.11E) and were defective in producing ROS (Fig. 3.11F). In contrast, mature follicles with *cut/hnt/ttk*^{RNAi} overexpression showed normal OA-induced Mmp2 activation (Fig. 3.11G). Overall, these results indicate that simultaneous upregulation of *hnt* and downregulation of *ttk69* is sufficient to rescue the defective OA/Oamb-Ca^{2+}-Mmp2 pathway in *cut*-overexpressing follicle cells, but not the defective OA/Oamb-Ca^{2+}-Nox-ROS pathway (Fig. 3.11H). Indirectly, these results also suggest that Cut influences additional factors in Nox-ROS production downstream of Ca^{2+} (Fig. 3.11H). In conclusion, our work suggests that downregulation of Cut in stage-14 follicle cells is essential for follicles to transition into full maturation, a preovulatory stage ready for ovulatory stimuli.

Discussion

Follicle cells undergo a third transition into stage 14 for ovulatory competence

Previous studies have focused on the two follicle cell transitions that occur in early and mid-oogenesis. The first transition occurs at stages 6/7, when follicle cells switch from the mitotic cycle to endocycle. The second transition happens at stages 10A/10B, as follicle cells complete endoreplication and shift into gene amplification

(Klusza and Deng, 2011). Together with our previous data, our results here demonstrate that follicle cells in stage-13 and stage-14 egg chambers differ in expression of multiple proteins, including Cut, Ttk69, Hnt, EcR.A, EcR.B1, Shd, Oamb, Mmp2. Therefore, follicle cells experience a novel third follicle cell transition when they develop into stage-14 egg chambers, and this transition is key to reach the final maturation, a state primed for ovulation/follicle rupture. In comparison to the two previous transitions, this final transition received little attention and no regulatory mechanism has been identified.

In this study, we illustrated the critical role of Cut downregulation at this final transition and characterized the epistatic relation between Cut and the rest of the known factors showing changes in expression at this transition. Ectopic Cut expression at stage 14 influences both Mmp2 activity and ROS production by regulating Ttk69, Hnt, and other unknown factors; however, ectopic Cut does not influence the ecdysteroid signaling at the stage 13/14 transition. Therefore, other signaling pathways are also involved in follicle cells to upregulate the ecdysteroid signaling during this transition. Alternatively, the ecdysteroid signaling may function upstream of Cut to downregulate Cut expression and promote this transition. Thus, the late follicle cell transition is a complex process in order to ensure proper maturation of the follicles for ovulation.

Recent work has started to unveil multiple signaling pathways that are required for *Drosophila* ovulation and are conserved in mammals. For example, Mmps show spatiotemporal activation in both *Drosophila* and mammalian mature follicles and are essential for ovulation (Curry and Osteen, 2003; Deady and Sun, 2015). ROS, particularly hydrogen peroxide, play essential roles for ovulation in *Drosophila* and mice

(Li et al., 2018; Shkolnik et al., 2011) and Hnt's role in ovulation can be replaced by human homolog Ras Response-element Binding protein 1 (Deady et al., 2017; Fan et al., 2009). Elevation of intracellular calcium in mature follicle cells either by OA/Oamb signaling in *Drosophila* or Luteinizing Hormone (LH) signaling in mammals seems important for ovulation (Breen et al., 2013; Deady and Sun, 2015). Paralleled to LH induced progesterone production prior to ovulation (Richards and Ascoli, 2018), stage-14 follicle cells upregulate the monooxygenase Shd to produce active steroid hormone for ovulation (Knapp and Sun, 2017). Thus, it stands to reason that the stage-13/14 transition follicle cells experience at the end of oogenesis may be fairly conserved.

Interaction between Cut, Hnt, and Ttk69 in Drosophila follicle cells

The transcription factors analyzed in this study (Cut, Hnt, and Ttk69) exhibit an intricate epistatic relationship throughout *Drosophila* oogenesis. In follicle cells during early oogenesis, upregulation of Hnt expression at stage 7 is required for suppressing Cut expression and transitioning from the mitotic cycle to the endocycle (Sun and Deng, 2007). Loss of Hnt leads to extended Cut expression into stage-7 follicle cells, while misexpression of Hnt in earlier follicle cells can drive premature downregulation of Cut. It is interesting to note that Cut knockdown in mitotic follicle cells also leads to premature upregulation of Hnt (Lo et al., 2019), indicating that Hnt and Cut antagonize each other to regulate the M/E switch. Hnt/Cut and Ttk69 do not regulate each other at the M/E transition (Sun and Deng, 2007); however, Ttk69 acts upstream of Hnt and Cut in the E/A switch during mid-oogenesis. Ecdysteroid-induced upregulation of Ttk69 expression leads to the suppression of Hnt and the induction of Cut expression in stage-

10B follicle cells (Sun et al., 2008). After stage-10B, Ttk69 also plays a crucial role in regulating dorsal appendage (DA) morphogenesis, as loss of Ttk69 in these stages causes defects in DA tube expansion (French et al., 2003). Ttk69 is required for follicle cells to take on proper cell fates and shapes during these stages, through regulation of multiple downstream factors, such as *broad*, *mirror*, *paxillin*, and *shibire*. Furthermore, expression of Ttk69 after stage-10B is also crucial to regulate the expression of numerous chorion genes and is thus critical for proper eggshell formation (Boyle and Berg, 2009; Peters et al., 2013). In this study we illustrate once again the opposing nature between Hnt and Cut in follicle cells during late oogenesis. Our work demonstrates that misexpression of Cut in stage-14 follicle cells can inhibit the re-upregulation of Hnt in these follicle cells, while Hnt knockdown in stage-14 follicle cells does not extend Cut expression. In addition, we found that misexpression of Cut in stage-14 follicle cells is able to induce expression of Ttk69, independent of Hnt. Our data suggest that Cut acts upstream of Hnt and Ttk69 during the late follicle cell transition. Depending on the different cellular environment, epistatic relationship among Cut, Hnt, and Ttk69 can change dramatically in the follicle cell lineage.

More work still needs to be done to understand what mechanism regulates induction of Hnt at stage 14. Induction of Hnt in stage 14 is not solely due to relief a transcriptional repression from Cut, as experiments knocking down Cut protein in follicle cells from stage 10B onwards are not sufficient to induce premature Hnt expression before stage 14 (Fig. 3.12). Thus, there must be another factor that is required to activate Hnt expression in stage-14 follicle cells, or multiple transcriptional repressors need to be relieved in order for Hnt to be turned on. It will be interesting to investigate

further the regulation of the complex signaling network necessary for follicle cells to transition into a stage-14 preovulatory state.

Materials & Methods

Drosophila genetics

Flies were reared on standard cornmeal and molasses food at 25°C, and experiments were performed at 29°C, unless noted otherwise. Two stage-14 follicle cell specific Gal4 drivers from the Janelia Gal4 collection (Pfeiffer et al., 2008) were used: *47A04-Gal4* and *44E10-Gal4*. *CG13083-Gal4* and *Oamb-RFP* are generated in this study. The following transgenic lines were used to knock down or overexpress genes in experiments: *UAS-cut* (a gift from Dr. Wu-Min Deng), *UAS-hnt* (Bloomington stock #5358), *UAS-ttk69* (*UAS-ttk69, ttk^{1e11}/TM3, Sb*; a gift from Dr. Celeste Berg), *UAS-ttk^{RNAi}* (Vienna stock #10855), *UAS-cut^{RNAi}* (Vienna stock #5687), *UAS-hnt^{RNAi}* (Vienna stock #3788). Isolation and identification of stage-14 follicles for *ex vivo* assays were performed using *44E10-Gal4* to drive *UAS-RFP* expression. The protein trap line *Mmp2::GFP* (Deady et al., 2015) was used for Mmp2 expression. Control flies for all experiments were prepared from crossing Gal4 drivers to Oregon-R flies.

CG13083-Gal4 and Oamb-RFP transgenes

To generate *CG13083-Gal4* driver, we amplified 896bp promoter region of CG13083, whose mRNA is detected in stage-12 follicle cells (Fakhouri et al., 2006), using Oregon-R genomic DNA and the following primer pair (5'-cacctcatggaaatcatatgcatcaac-3' and 5'-cccgctgagtgtctcttttc-3'). This fragment was

inserted into pENTR™-vector (Invitrogen) and subcloned into *pBPGUw* vector (Addgene) by LR Clonase™ II enzyme mix (Invitrogen) to generate the final construct *CG13083-Gal4*. To generate the *Oamb-RFP* reporter, we amplified 803bp promoter region of *Oamb* gene, the same fragment used to generate *47A04-Gal4* driver, using the following primer pair (5'-TggggtaccccAACTGGCCAGAACTAACGGTTC-3' and 5'-TTcgggatcccGCCGGGTTTTGTGAAATTAATAG-3'). The fragment were then digested with KpnI and BamHI (NEB) and inserted into *pRed H-Pelican* vector (DGRC) to generate *Oamb-RFP* vector. Both vectors were injected into fly embryos to generate the transgenic *CG13083-Gal4* and *Oamb-RFP* stocks by the BestGene through standard procedure.

Ovulation assays

Egg laying and mature follicle analyses were performed as previously described (Deady and Sun, 2015). Five-day old females fed with wet yeast for one day were placed with Oregon-R males (5 females: 10 males) in one bottle for egg laying on molasses plates over two days in 29°C (with removal and replacement of plates every 22 hours). Ovaries were dissected out after egg laying, and mature follicles within each ovary pair were quantified.

Ex vivo follicle rupture, gelatinase assay, and qRT-PCR

The *ex vivo* follicle rupture assays were performed as described before (Knapp et al., 2018). Ovaries from six-day old virgin females fed with wet yeast for three days were dissected out and stage-14 follicles were isolated in Grace's insect medium (Caisson

Labs). Isolated follicles were divided into groups of ~30 follicles, then cultured for three hours in culture media along with 20μM OA (Sigma) or 5μM ionomycin (Cayman Chemical) in 29 °C. Each data point represents the percent of ruptured follicles per experimental group. Data were shown as mean percentage ± standard deviation (s.d.).

In situ zymography to detect gelatinase activity was performed as described previously (Knapp et al., 2018). Mature follicles were cultured for three hours with 25 μg/mL of DQ-gelatin conjugated with fluorescein (Invitrogen) and 20μM OA. The number of mature follicles with posterior fluorescein signal was counted afterwards, and data represented as percent of mature follicles with posterior fluorescein signal. Data were shown as mean percentage ± s.d.

For qRT-PCR, 4-6 day old virgin females fed with wet yeast for three days were used to isolate mature follicles according to *44E10-Gal4 driving UAS-RFP* expression. For experiments where quantification was performed in either posterior or anterior halves, mature follicles were first isolated and cut in half with the Vannas-Tubingen spring scissors (Fine Science Tools, Item No. 15003-08) in cold Grace's medium. Anterior/posterior halves were separated into different tubes in batches of 30. Total RNAs were extracted using Direct-zolTM RNA MicroPrep Kit (Zymo Research) from 60 stage-14 follicles or follicle halves, and cDNA synthesis and real-time PCR amplification with three technical repeats were performed as previously described (Knapp and Sun, 2017; Li et al., 2018). Data from one representative biological replicate were presented

as mean ± s.d. and two to three biological replicates were performed to ensure reproducibility.

Superoxide detection

Measurement of superoxide production was performed as previously described (Li et al., 2018). Thirty mature follicles were isolated and placed in each well of a 96-well plate with 250µl of culture media with 200µM of L-012 (Wako Chemicals). Plates were placed in a synergy H1 plate reader (BioTek Instruments, Inc.) for a 45-minute L-012 luminescence reading. At five minutes, 20µM of OA was added to each well. Three to four wells (technical repeats) were used in each experiment for each genotype, and mean ± s.d. of the technical repeats was calculated. Each experiment was repeated at least twice.

Immunostaining and microscopy

Immunostaining was performed following a standard procedure comprising ovary dissection, fixation in 4% EM-grade paraformaldehyde for 13 min, blocking in PBTG (PBS + 0.2% Triton +0.5% BSA +2% normal goat serum), and primary and secondary antibody staining diluted in PBTG. The following primary antibodies were used: mouse anti-Cut (1:15), anti-Hnt (1:75), anti-EcR.A (1:15), anti-EcR.B1 (1:15) antibodies from Developmental Study Hybridoma Bank; Rabbit anti-Ttk69 (1:100; a gift from Dr. Wanzhong Ge) (Ge et al., 2015), anti-GFP (1:4000; Invitrogen), anti-Shd (1:250; a gift from Dr. Michael O'Connor). Alexa-488 and Alexa-568 goat secondary antibody (1:1000; Invitrogen) were used as secondary antibodies. Images were acquired using a Leica TCS SP8 confocal microscope or Leica MZ10F fluorescent stereoscope with a

sCOMS camera (PCO.Edge) and assembled using Photoshop software (Adobe, Inc) and ImageJ.

Statistical analysis

Statistical tests were performed using Prism 7 (GraphPad, San Diego, CA). Quantification results were presented as mean ± s.d., and statistical analysis was conducted using Student's *t*-test. For comparison of more than two means, one-way ANOVA with *post hoc* Fisher's Least Significant Difference test was used. For comparison of distribution, Chi square test was used.

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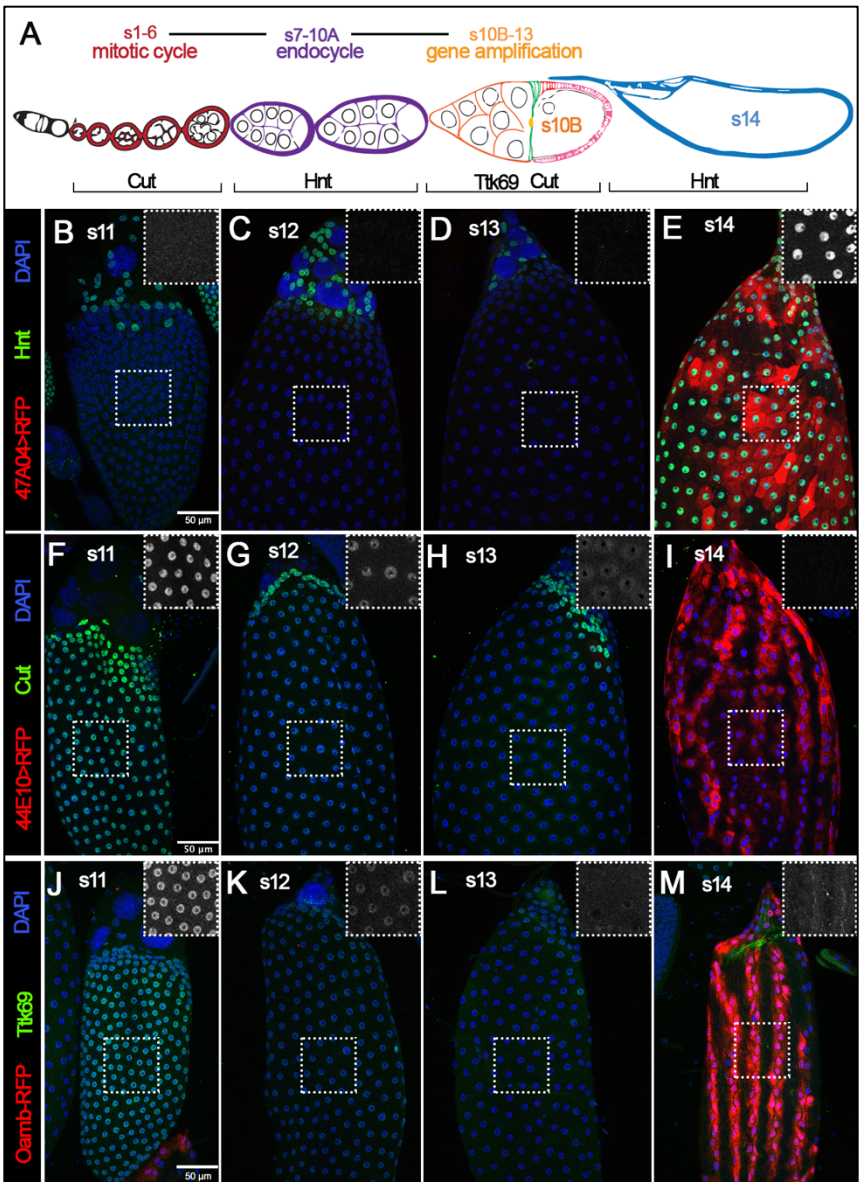

Figure 3.1. The expression pattern of several transcription factors in late oogenesis of Drosophila

(A) A cartoon showing the follicle cell transitions in *Drosophila* oogenesis. Follicle cells are in the mitotic cycle during stages 1-6 (dark red), the endocycle during stages 7-10A (purple), and gene amplification during stages 10B-13. At stage 10B, follicle cells have differentiated into stretch follicle cells (orange), border cells (yellow), centripetal follicle cells (green), dorsal appendage cells (red), and mainbody follicle cells (pink). The expression pattern of several transcription factors throughout oogenesis is outlined as well. (B-E) The expression of Hnt in late oogenesis. The stage-14 follicle is marked by *47A04-Gal4* driving *UAS-RFP* expression. The insets are higher magnification of Hnt expression (white) in outlined areas. The DAPI is used to label cell nuclei in blue through figures. (F-M) The expression of Cut (F-I) and Ttk69 (J-M) in late oogenesis. The stage-14 follicles are marked by 44E10>RFP (red in F-I) or Oamb-RFP (red in J-M) expression. Scale bars: 50 microns.

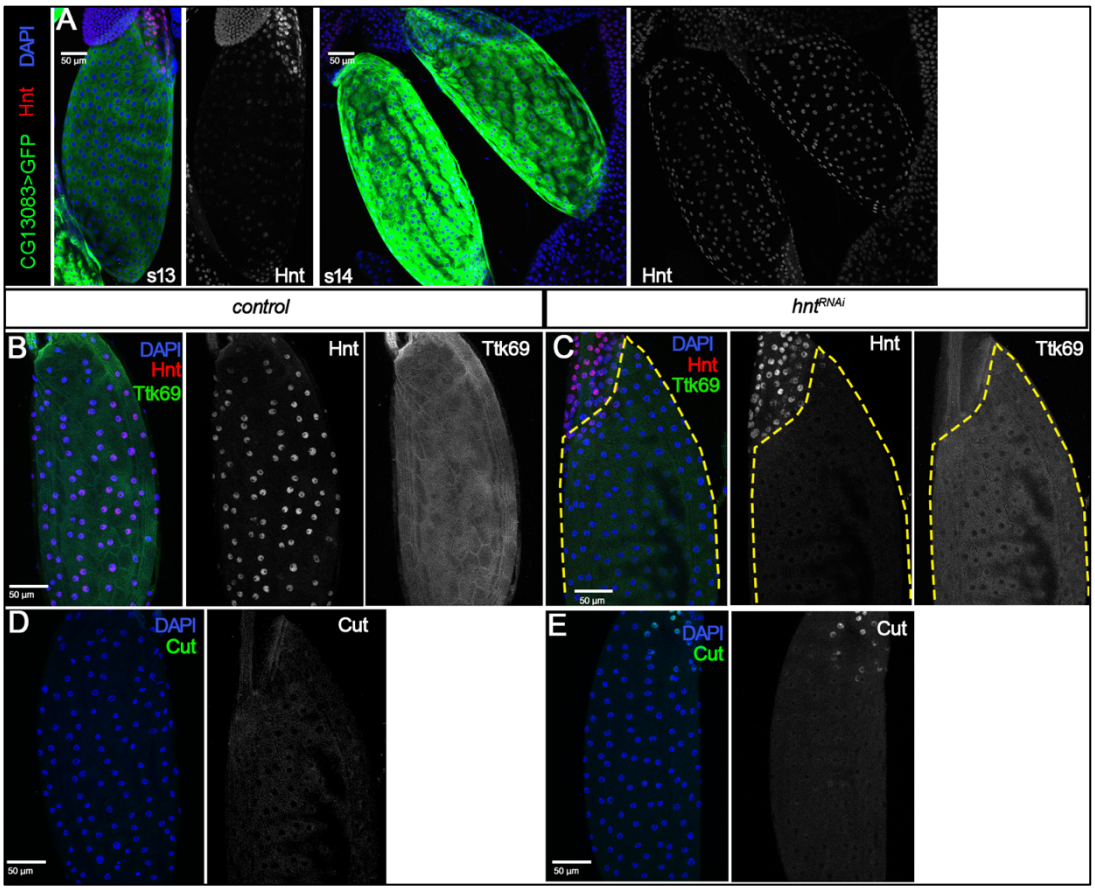

Figure 3.2. Hnt knockdown in stage-14 follicle cells is not sufficient to extend Cut or Ttk69 expression.

(A) Representative images show expression pattern of CG13083-Gal4 (green) with *CG13083-Gal4* driving *UAS-GFP* in stage-13 and stage-14 main body follicle cells. The stage-14 egg chambers are marked by expression of Hnt (red) in main body follicle cells. (B-C) Representative images show expression of Hnt (red) and Ttk69 (green) in stage-14 follicle cells of control (B) or *CG13083-Gal4>hnt*^{RNAi} (C) egg chambers, main body follicle cells are outlined in yellow. (D-E) Representative images show expression of Cut (green) in stage-14 follicle cells of control (D) and *CG13083-Gal4>hnt*^{RNAi} (E) egg chambers. (C,E) In *CG13083-Gal4>hnt*^{RNAi} over 96% of stage-14 follicles examined displayed knockdown of Hnt in mainbody follicle cells, and 100% of stage-14 follicles demonstrated no extended expression of Ttk69 or Cut (n=30). Scale bars: 50 microns

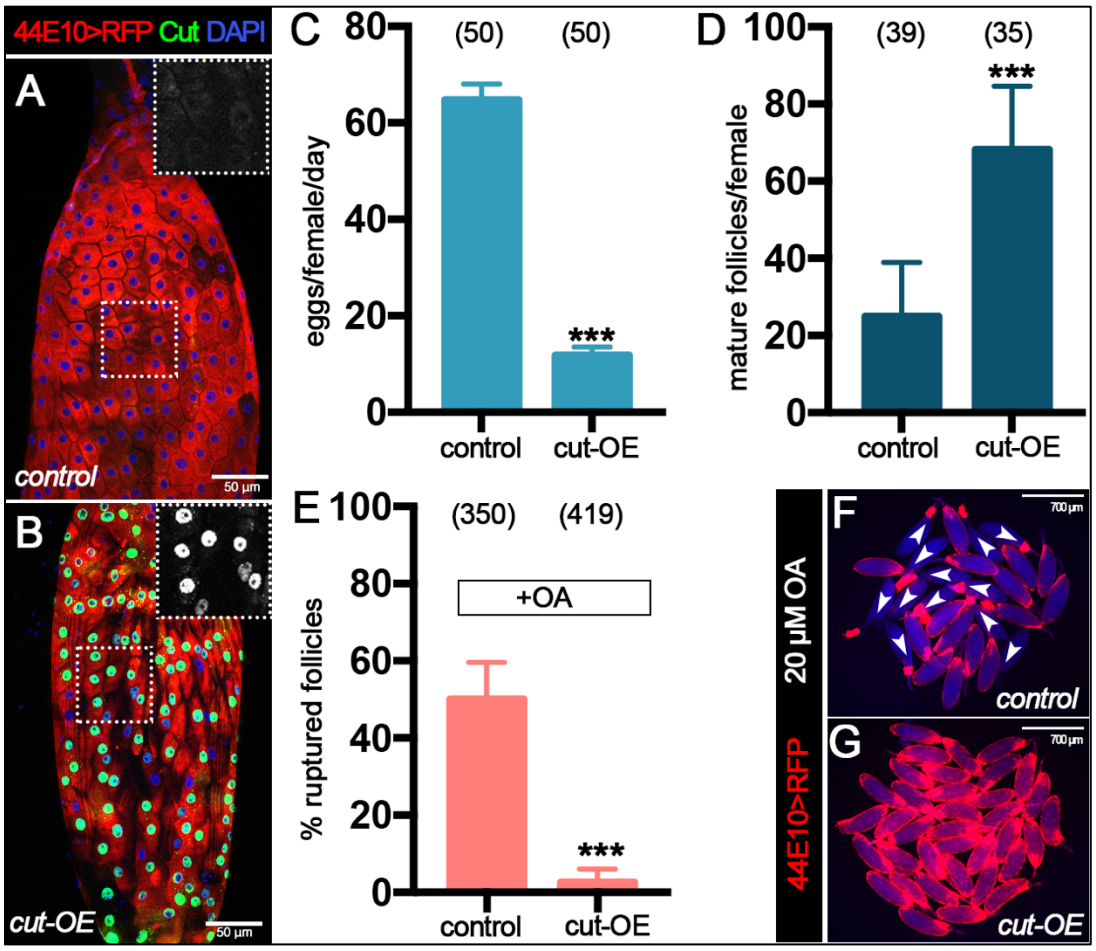

Figure 3.3. Ectopic Cut in stage-14 follicle cells blocks ovulation and follicle rupture.

(A-B) Representative images (n = 30) show Cut protein (green) in control (A) and *cut-overexpressing* (*cut-OE*) (B) follicles driven by *44E10-Gal4*. Stage-14 follicle cells are marked by 44E10>RFP (red). Insets show Cut expression (white) in outlined areas. (C-D) Quantification of egg laying (C) and mature follicles in each female's ovaries post egg laying (D) in control or *cut-OE* females with *44E10-Gal4*. The number of females is noted above each bar. (E) Quantification of OA-induced follicle rupture. The mature follicles were isolated according to 44E10>RFP expression and the number of mature follicles analyzed is noted above each bar. (F-G) Representative images show control (F) and *cut-OE* (G) mature follicles after the three-hour culture with OA. Ruptured follicles are marked by arrowheads. Scale bars: 50 microns. All data are plotted as mean ± s.d. Statistics: t-test. Asterisk representation: p-value <0.001 (***).

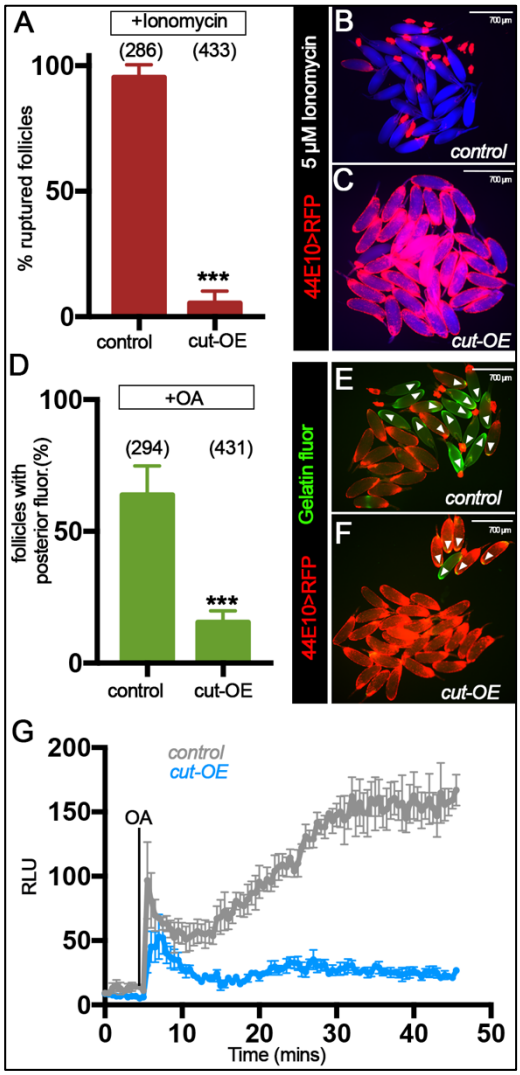

Figure 3.4. Ectopic Cut in stage-14 follicle cells disrupts Mmp2 activity and ROS production.

(A) Quantification of ionomycin-induced follicle rupture. The number of mature follicles analyzed is noted above each bar. (B-C) Representative images show control (B) and *cut-OE* (C) mature follicles with *44E10-Gal4* after the three-hour culture with ionomycin. (D) Quantification of mature follicles with posterior green fluorescence (indicating gelatinase activity) after the three-hour culture with OA. (E-F) Representative images showing gelatinase activity (green) in control (E) and *cut-OE* (F) mature follicles with *44E10-Gal4* after the three-hour culture with OA. Mature follicles with posterior gelatinase activity are marked by arrowheads. (G) Quantification of L-012 luminescent signal (indicating superoxide production) in control (grey) and *cut-OE* (blue) mature follicles with *44E10-Gal4*. OA is added at the 5-minute time point. RLU: relative luminescence unit. All data are plotted with mean ± s.d. indicated. Statistics: t-test. Asterisk representation: p-value <0.001 (***).

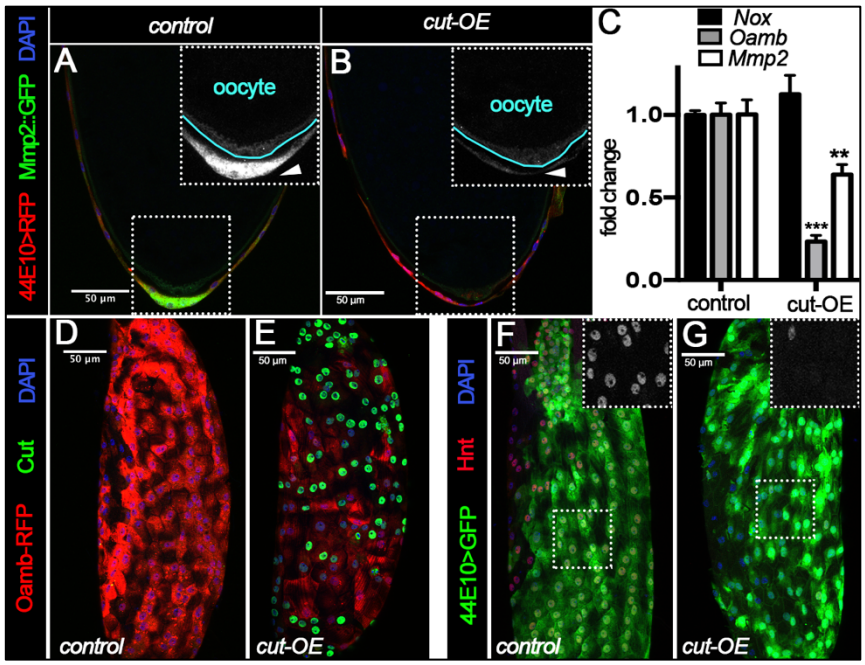

Figure 3.5. Ectopic Cut in stage-14 follicle cells disrupts the expression of Mmp2, Oamb, and Hnt

(A-B) Representative images (n = 30) show Mmp2::GFP expression (green) in control (A) or *cut-OE* (B) mature follicles with *44E10-Gal4.* Stage-14 follicle cells are marked by 44E10>RFP (red). Insets are high magnification of Mmp2::GFP expression (white) in squared areas. Arrowheads point to posterior follicle cells, and oocytes are outlined in cyan. (C) qRT-PCR measurement of *Nox*, *Oamb,* and *Mmp2* mRNA levels in control or *cut-OE* mature follicles with *44E10-Gal4*. (D-E) Representative images (n = 30) show Oamb-RFP expression (red) in control (D) or *cut-OE* (E) mature follicles with *44E10-Gal4*. Cut protein is in green. (F-G) Representative images (n = 30) show Hnt protein expression (red) in control (F) and *cut-OE* (G) mature follicles with *44E10-Gal4* (green). Follicle cells are marked by 44E10>GFP, and insets are high magnification of Hnt expression (white) in squared areas. Scale bars: 50 microns. All data are plotted with mean ± s.d. indicated. Statistics: t-test. Asterisk representation: p-value <0.01 (**) and p-value <0.001 (***).

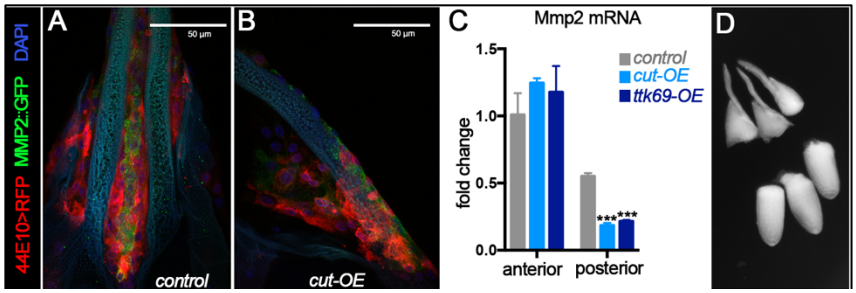

Figure 3.6. Ectopic Cut does not disrupt Mmp2 expression in anterior follicle cells.

(A-B) Representative images show Mmp2::GFP expression (green) in anterior follicle cells in control (A) and *cut-overexpressing* (*cut-OE*) (B) egg chambers with *44E10-Gal4* (red). (C) qRT-PCR measurement of *Mmp2* mRNA levels in control, *cut-OE*, and *ttk69-OE* stage-14 follicles with *44E10-Gal4*. Stage-14 follicles were cut in half and measurements for *Mmp2* mRNA levels were performed separately in either posterior or anterior halves. (D) A representative image shows stage-14 follicles after they were cut in half. All data are plotted with mean ± s.d. indicated. Statistics: t-test. Asterisk representation: p-value <0.001 (***).

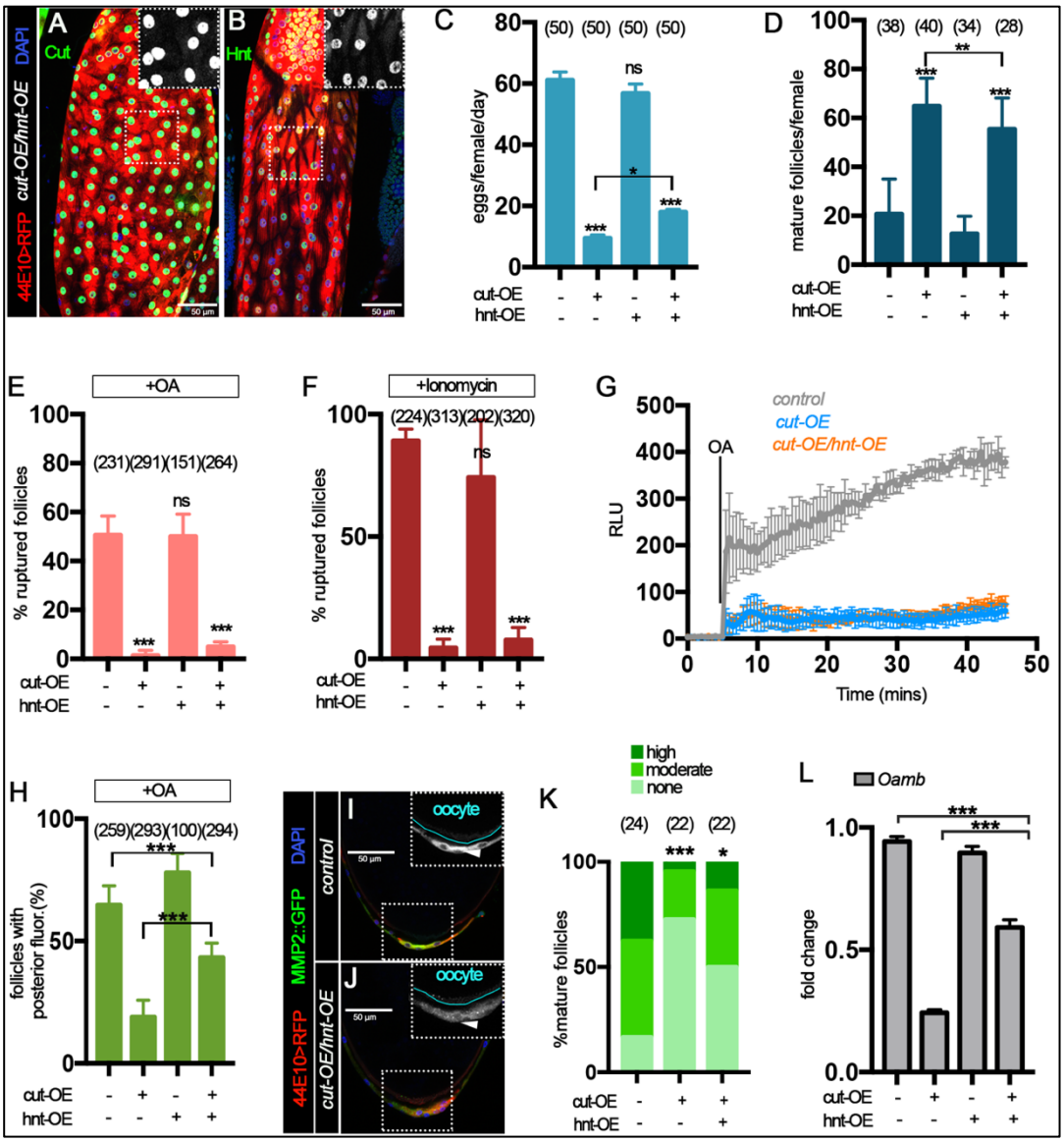

Figure 3.5. Hnt cannot fully rescue ovulation defects caused by ectopic Cut.

(A-B) Representative images (n = 30) show both Cut (A) and Hnt (B) expression in mature follicles with *cut/hnt overexpression (cut-OE/hnt-OE)* driven by *44E10-Gal4*. Insets are high magnification of Cut (A) or Hnt (B) expression in squared areas. (C-D) Quantification of egg laying (C) and mature follicles in each female after egg laying (D). (E-F) Quantification of follicle rupture, stimulated with OA (E) or ionomycin (F). (G) Quantification of L-012 luminescent signal (indicating superoxide production) in mature follicles stimulated with OA at the 5-minute time point. (H) Quantification of follicles with posterior green fluorescence (indicating gelatinase activity) after a three-hour OA stimulation. (I-J) Representative images show Mmp2::GFP expression (green) in control (I) or *cut-OE/hnt-OE* (J) mature follicles with *44E10-Gal4*. Insets are high magnification of Mmp2::GFP expression (white) in squared areas, arrowheads point to posterior follicle cells, and oocytes are outlined in cyan. (K) Quantification of Mmp2::GFP expression in control, *cut-OE*, and *cut-OE/hnt-OE* mature follicles with *44E10-Gal4*. (L) qRT-PCR measurement of *Oamb* mRNA levels in mature follicles isolated according to 44E10>RFP expression.

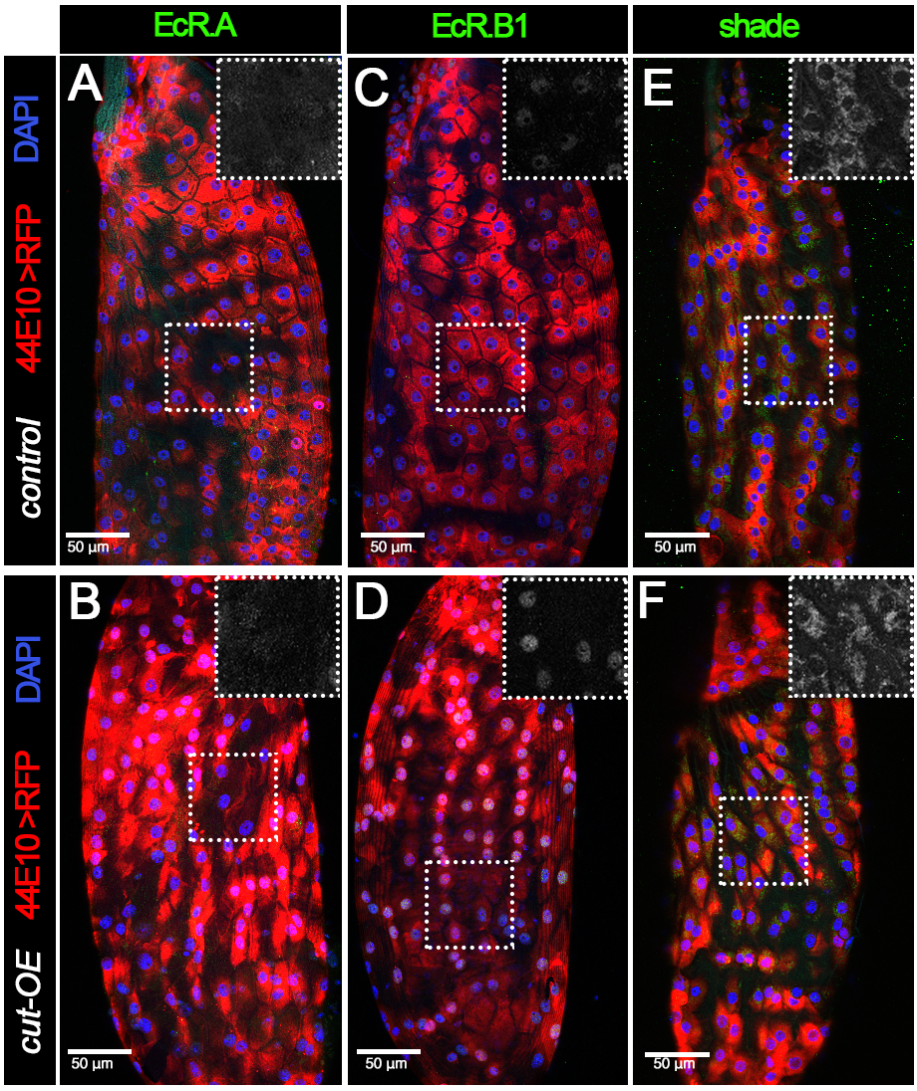

Figure 3.6. Ectopic Cut does not affect EcR.A, EcR.B1, or Shd expression.

(A-B) Representative images show EcR.A expression (green) in control (A) or *cut-OE* (B) follicles with *44E10-Gal4*. Stage-14 follicle cells are marked by 44E10>RFP (red), and insets are high magnification of EcR.A expression in squared areas. (C-D) Representative images show EcR.B1 expression (green) in control (C) or *cut-OE* (D) stage-14 follicles. (E-F) Representative images show Shd expression (green) in control (E) or *cut-OE* (F) stage-14 follicles.

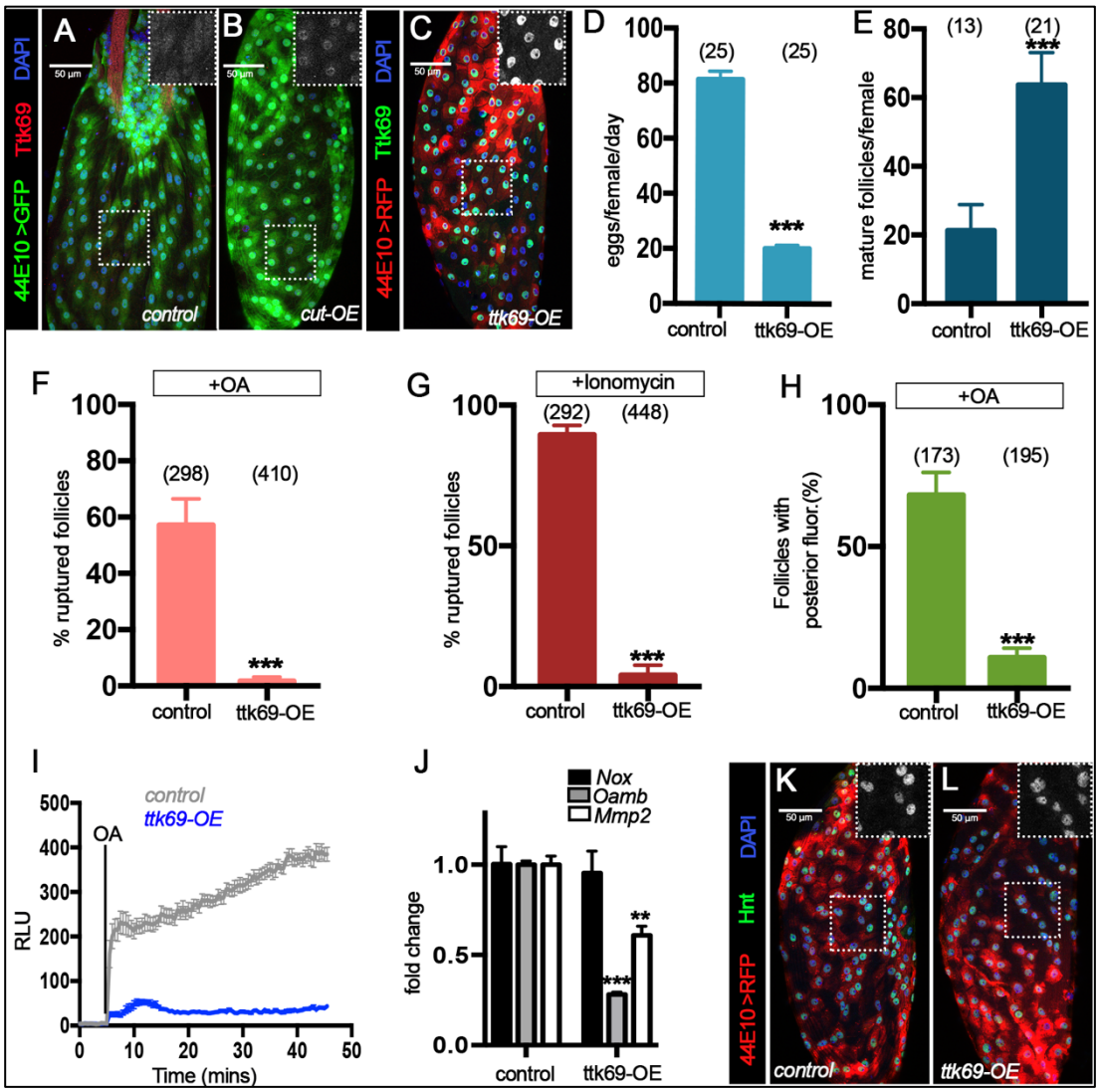

Figure 3.7. Ectopic Cut in stage-14 follicle cells promotes Ttk69 expression, which disrupts ovulation and follicle rupture.

(A-B) Representative images (n = 30) show Ttk69 expression (red) in control (A) or *cut-OE* (B) mature follicles with *44E10-Gal4*. (C) Representative image (n = 30) shows Ttk69 expression (green) in *ttk69-overexpressing (ttk69-OE)* mature follicles with *44E10-Gal4*. Stage-14 follicle cells are marked by 44E10>RFP (red). Insets are high magnification of Ttk69 expression (white) in squared areas. (D-E) Quantification of egg laying (D) and number of mature follicles per female after 2 days of egg laying (E) in control or *ttk69-OE* females. (F-G) Quantification of follicle rupture through either OA stimulation (F) or ionomycin stimulation (G). (H) Quantification of follicles with posterior green fluorescence (indicating gelatinase activity) following OA stimulation. (I) Quantification of L-012 luminescent signal (indicating superoxide production) in mature follicles stimulated with OA at the 5-minute time point. (J) qRT-PCR measurement of *Nox*, *Oamb*, and *Mmp2* mRNA levels. (K-L) Representative images (n = 30) show Hnt expression (green) in control (K) or *ttk69-OE* follicles (L) with *44E10-Gal4*. Scale bars: 50 microns. All data are plotted with mean ± s.d. indicated. Statistics: t-test. Asterisk representation: p-value <0.01 (**) and p-value <0.001 (***).

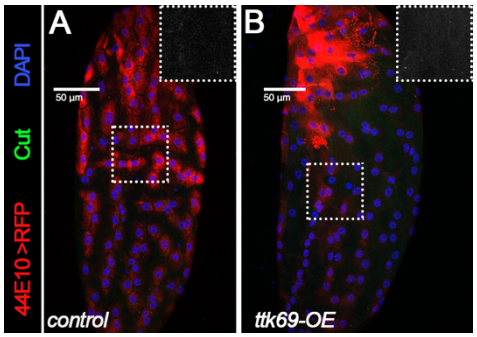

Figure 3.10. Ectopic Ttk69 does not induce the expression of Cut in stage-14 follicle cells.

(A-B) Representative images show expression of Cut (green) in stage-14 follicle cells of control (A) or *ttk69-OE* (B) follicles with *44E10-Gal4*.

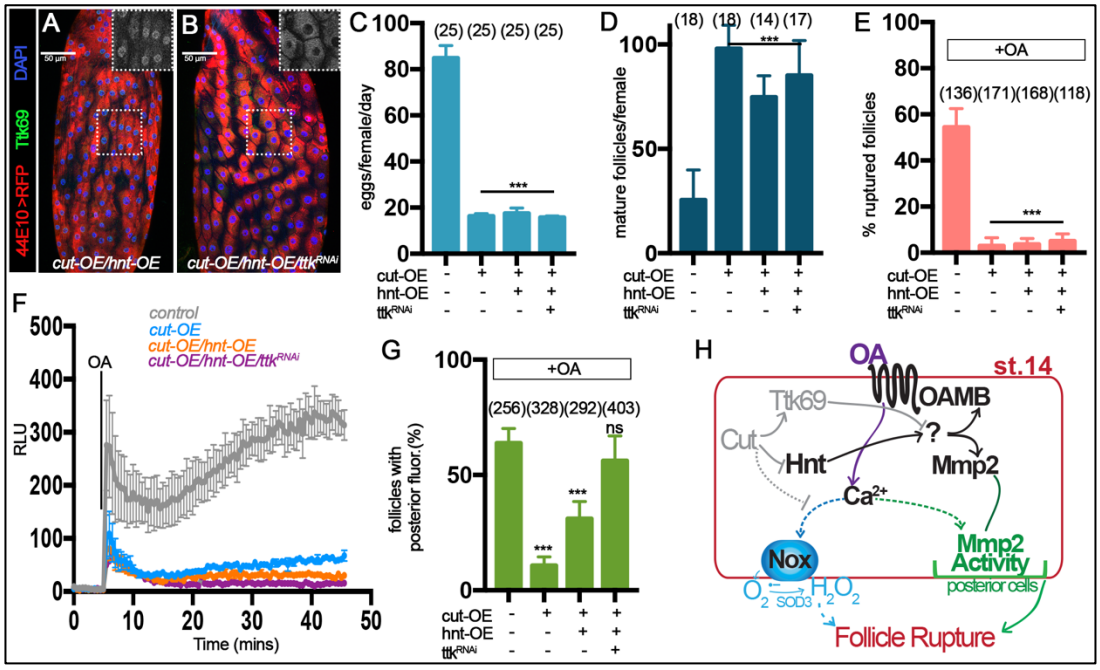

Figure 3.11. Overexpression of Hnt and knockdown of Ttk69 fully rescue Mmp2 activation in cut-overexpressing follicles.

(A-B) Representative images (n = 30) show Ttk69 expression (green) in *cut-OE/hnt-OE* (A) or *cut/hnt/ttk*^{RNAi}*-overexpressing* (*cut-OE/hnt-OE/ttk*^{RNAi}) (B) mature follicles with *44E10-Gal4*. Insets are high magnification of Ttk69 expression (white) in squared areas. (C-D) Quantification of egg laying (C) and mature follicles (D). (E) Quantification of OA-induced follicle rupture in mature follicles with *44E10-Gal4* driving *cut-OE*, *hnt-OE,* and/or *ttk*^{RNAi}. (F) Quantification of L-012 luminescent signal (indicating superoxide production) in mature follicles with *44E10-Gal4* driving *cut-OE*, *hnt-OE,* and/or *ttk*^{RNAi} stimulated with OA at the 5-minute time point. (G) Quantification of mature follicles with posterior green fluorescence (indicating gelatinase activity) after three-hour OA stimulation. (H) A schematic diagram shows the regulation of stage 13/14 transition. Ectopic expression of Cut (gray) in stage-14 follicle cells disrupts follicle rupture competency by inhibiting upregulation of Hnt and promoting ectopic expression of Ttk69 (gray).

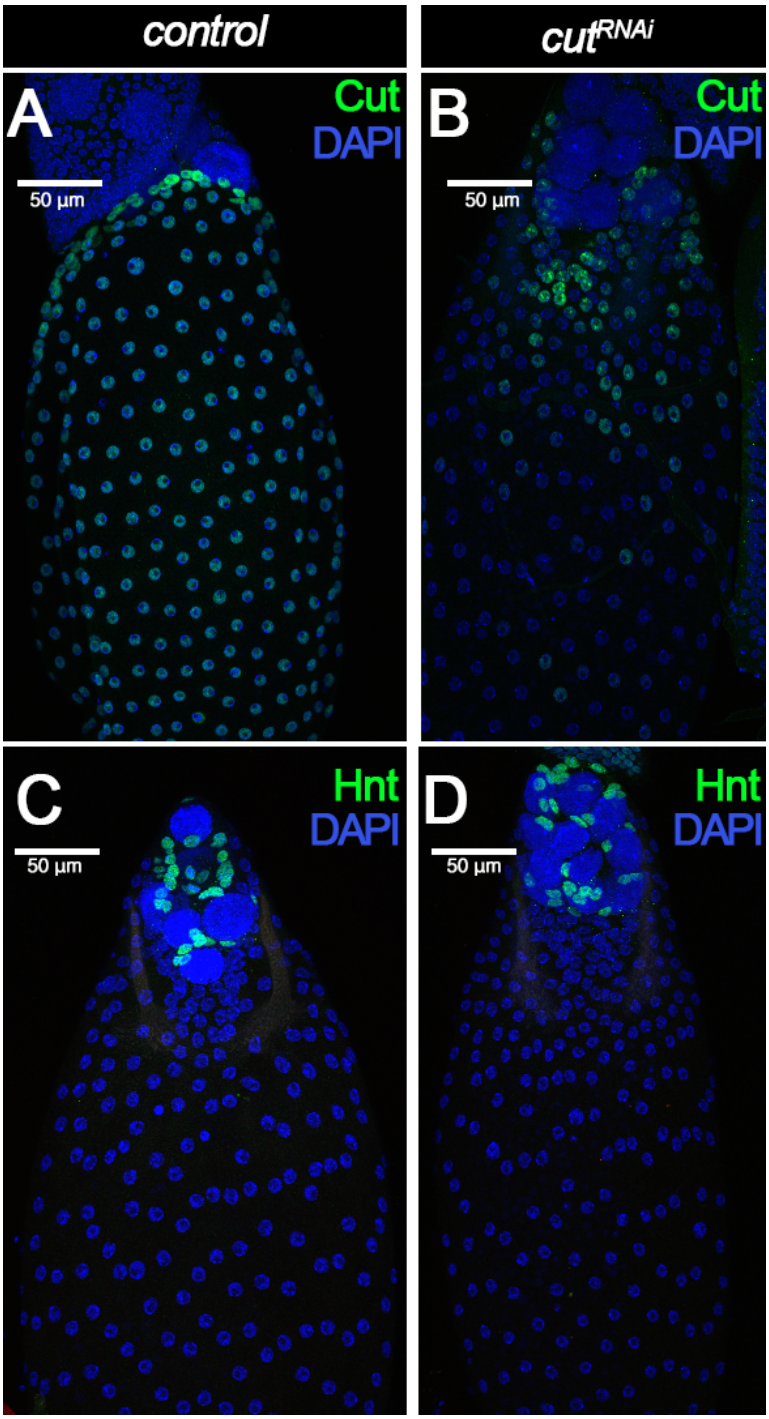

Figure 3.8.Cut knockdown in follicle cells during late oogenesis is not sufficient to induce premature Hnt upregulation.

(A-B) Representative images show Cut expression (green) in stage-12 follicle cells in the control egg chamber(A) and the egg chamber with *VM26Aa-Gal4 driving cut*^{RNAi} (*VM26Aa>cut*^{RNAi}; B). Cut expression is dramatically knocked down. (C-D) Representative images show Hnt expression (green) in control (C) and *VM26Aa>cut*^{RNAi} (D) stage-12 egg chambers. Hnt is not detected in mainbody follicle cells.

Chapter 4 : The NR5A Nuclear Receptor Ftz-f1 Promotes Follicle Maturation and Ovulation via bHLH Transcription Factor Single-Minded

Introduction

Female fertility, an essential half of the reproductive equation, requires proper follicle maturation and ovulation. The NR5A family of nuclear receptors are critical for the success of these complex ovarian processes across species (Jeyasuria et al., 2004; Meinsohn et al., 2019; Mlynarczuk et al., 2013; Sun and Spradling, 2013; Suresh and Medhamurthy, 2012). The majority of what is known concerning these NR5A receptors in female fertility stems from studies performed over the past two decades in rodent models. These investigations have shown that both members of this family, NR5A1 (steroidogenic factor-1 or SF-1) and NR5A2 (liver receptor homolog-1 or LRH-1), are expressed in the follicle cells that encapsulate the oocyte throughout oogenesis (Falender et al., 2003; Hinshelwood et al., 2003). Follicle cell specific loss of either receptor leads to drastically impaired fertility. LRH-1 knockout in granulosa cells in either primary or more developed antral follicles results in severe anovulation, which are attributed to the inhibition of cumulus expansion, upregulation of steroidal biosynthetic genes, and granulosa cell proliferation/differentiation (Bertolin et al., 2014, 2017; Bianco et al., 2019; Duggavathi et al., 2008; Meinsohn et al., 2018). Targeted depletion of SF-1 in granulosa cells of primary follicles has shown to result in hypoplastic ovaries and a dramatically reduced number of developing follicles (Pelusi et al., 2008a). Much less is known about the molecular mechanism of SF-1 in these ovarian follicle cells.

SF-1 was initially recognized as the mammalian homolog of the *Drosophila fushi tarazu-factor 1* (*ftz-f1*), which regulates the expression and function of the pair-rule segmentation gene *fushi tarazu* (*ftz*) during early embryogenesis (Lala et al., 1992; Ueda et al., 1990). With different promoter usage, the *ftz-f1* gene encodes two protein isoforms (α and β), each comprised of unique N-terminal sequences and common C-terminal sequences (Lavorgna et al., 1991, 1993). αFtz-f1 is expressed during early embryogenesis and involved in regulating *ftz* expression, whereas βFtz-f1 is not detected until late embryogenesis and arising again in subsequent larval and prepupae stages (Broadus et al., 1999; Lavorgna et al., 1993; Yamada et al., 2000). βFtz-f1 is induced in between ecdysone pulses and required for embryogenesis, larval ecdysis, and pupal development (Bond et al., 2011; Boulanger et al., 2011; Broadus et al., 1999; Cho et al., 2014; Fortier et al., 2003; Sultan et al., 2014; Yamada et al., 2000). During these developmental stages, βFtz-f1 is thought to function as a biological timer to induce the next ecdysone pulse through regulating ecdysteroid synthesis genes (Akagi et al., 2016; Parvy et al., 2005; Talamillo et al., 2013). In addition, Ftz-f1 has been found to function as an oncogene and promote tumorigenesis and tumor invasiveness in *Drosophila* imaginal discs (Kulshammer et al., 2015; Atkins et al., 2016; Song et al., 2019). Even though initial studies demonstrated the potential for Ftz-f1 activity in adult tissues (Ueda et al., 1990), little has been done to study what roles Ftz-f1 plays in adult flies, particularly in oogenesis.

Drosophila oogenesis is an excellent model for studying many cell biology questions in the last few decades. *Drosophila* oogenesis occurs in the ovariole, ~16 of which bundle together to form an ovary. At the anterior tip of the ovariole, germline and

follicle stem cells proliferate to produce daughter cells to form a stage-1 egg chamber (also named follicle in this paper), which develop through 14 morphologically distinct stages into a stage-14 egg chamber (also named mature follicle; (Spradling, 1993) and other reviews). Each follicle contains a layer of somatic follicle cells encasing 16 interconnected germ cells, one of which differentiates into an oocyte, while the rest of which function as nurse cells to support oocyte growth and are eventually degraded in mature follicles. Somatic follicle cells proliferate at stages 1-6 and transition into endoreplication at stages 7-10A induced by Notch signaling (Deng et al., 2001; Shcherbata et al., 2004). At stage 10B, a pulse of ecdysone signaling induces follicle cell transition from endoreplication to synchronized gene amplification, which is critical for eggshell production, via zinc-finger transcription factor Ttk69 (Sun et al., 2008). This is also accompanied by the downregulation of the zinc-finger transcription factor Hindsight (Hnt) and the upregulation of the homeodomain transcription factor Cut in stage-10B follicle cells (Sun et al., 2008). As follicles develop from stage 10B onwards, Ttk69 and Cut downregulate and by stage 14 another critical follicle cell transition occurs: Hnt is re-upregulated in follicle cells while Cut and Ttk69 are completely lost (Knapp et al., 2019). This transition is critical for the follicle to gain ovulatory competency via upregulation of Octopamine receptor in mushroom body (Oamb) and Matrix metalloproteinase 2 (Mmp2) (Deady and Sun, 2015; Deady et al., 2015, 2017; Knapp et al., 2019). In addition, follicle cells at stage 14 upregulate NADPH oxidase (Nox) expression, downregulate EcR.B1 and EcR.A, and receive another pulse of ecdysone signaling via EcR.B2 to become ovulatory competent (Knapp and Sun, 2017;

Li et al., 2018). However, it is largely unknown how follicle cells differentiate from stage 10B to stage 14.

In this study, we demonstrate that Ftz-f1 is transiently expressed in *Drosophila* follicle cells at stages 10B-12 and this expression is induced by ecdysteroid signaling in stage-10B follicle cells, independent of Ttk69. Loss of *ftz-f1* in follicle cells after stage 10B severely inhibits follicle cell differentiation into the maturation stage, resulting in follicles incompetent for follicle rupture and ovulation. In addition, we identify the transcription factor Single-Minded (Sim), whose functions are known in the central nervous system development (Crews, 1988; Muralidhar et al., 1993; Nambu et al., 1990; Thomas et al., 1988), as a novel target of Ftz-f1 in follicle cells. We demonstrate that Sim functions downstream of Ftz-f1 for follicle cell differentiation/maturation. Furthermore, we demonstrate the role of Ftz-f1 in follicle cell maturation is functionally conserved as ectopic expression of mouse SF-1 is able to rescue Ftz-f1 functionality in this process. These findings demonstrate a more conserved role of NR5A nuclear receptors in *Drosophila* and mammalian reproduction and help elucidate potential mechanisms downstream of NR5A nuclear receptor signaling required for female fertility across species.

Results

Ftz-f1 expression is induced in stage-10B follicle cells through ecdysone signaling

To investigate the role of Ftz-f1 in female fertility, we first analyzed the expression of Ftz-f1 throughout oogenesis using anti-Ftz-f1 antibody. Ftz-f1 protein is not detected in germline cells and ovarian follicle cells from stage 1 to stage 10A (Fig.

4.1A); however, it is drastically upregulated in all follicle cells at stage 10B (Fig. 4.1B), when follicle cells transition into synchronized gene amplification. Following stage 10B, Ftz-f1 begins to progressively decrease in follicle cells (except anterior stretch follicle cells) and is no longer detectable in stage-13/14 follicle cells (Fig. 4.1C-F). A *ftz-f1::GFP* transgene showed that the expression of Ftz-f1::GFP tagged protein completely matches Ftz-f1 antibody staining (Fig. 4.2A-E). In addition, we also examined the *ftz-f1* transcription using the enhancer trap line *ftz-f1* $^{fs(3)2877}$, which has a P-element containing lacZ gene inserted in the *ftz-f1* gene (Karpen and Spradling, 1992). Expression of βGal is also induced in stage-10B follicle cells and stays high in stage-13/14 follicle cells (Fig. 4.2F-J), which is likely a result of βGal not being subjected to endogenous protein regulation. Together, our data suggests that both *ftz-f1* mRNA and protein are transiently induced in stage-10B to12 follicle cells during *Drosophila* oogenesis.

The transition from endoreplication to gene amplification at stages 10A/10B is induced through ecdysone signaling, which carries out its function via inducing upregulation of the zinc-finger transcription factor Ttk69 (Sun et al., 2008). Considering the fact that *ftz-f1* is an ecdysone-induced gene during development, we examined whether ecdysone signaling regulates *ftz-f1* expression in follicle cells. Using the flip-out Gal4 system (Pignoni and Zipursky, 1997), we disrupted the ecdysone signaling via misexpression of a dominant-negative (DN) form of ecdysone receptor (EcR^{DN}) (Cherbas et al., 2003). EcR^{DN}-overexpressing follicle cells showed a complete loss of Ftz-f1 in stage-10B egg chambers (Fig. 4.1G), indicating that Ftz-f1 expression is induced by ecdysone signaling.

To determine whether Ftz-f1 is induced by Ttk69, the downstream target of ecdysone signaling, we knocked down Ttk69 expression by overexpressing ttk^{RNAi} in the flip-out clones. Follicle-cell clones with ttk^{RNAi} overexpression showed no detectable Ttk69 (Fig. 4.3A) but normal Ftz-f1 expression in stage-10B egg chambers (Fig. 4.1H). To determine whether Ftz-f1 regulates Ttk69 expression, we generated $ftz\text{-}f1^{ex7}$ mutant clones using the MARCM system (Wu and Luo, 2006). *ftz-f1* mutant follicle cells exhibit normal expression of Ttk69 (Fig. 4.1I). In addition, *ftz-f1* mutant follicle cells properly transitioned into the gene amplification stage in stage-10B egg chambers according to punctate EDU staining (Fig. 4.1J). Our results indicate that ecdysone signaling induces both Ftz-f1 and Ttk69 upregulation in stage-10B follicle cells; the latter one leads to the endoreplication/gene amplification transition, while the former one does not.

Transient expression of Ftz-f1 in late oogenesis is required for ovulation and follicle rupture

To determine the function of Ftz-f1 in follicle cells, we knocked down *ftz-f1* expression in follicle cells using *Vm26Aa-Gal4*, which starts to express in columnar follicle cells at stage 10B (Peters et al., 2013). Both $ftz\text{-}f1^{RNAi1}$ and $ftz\text{-}f1^{RNAi2}$ showed efficient knockdown of *ftz-f1* in stage-10B and stage-12 follicle cells when driven by *Vm26Aa-Gal4* (Fig. 4.4A-C, Fig. 4.5A-C), although $ftz\text{-}f1^{RNAi1}$ is more efficient than $ftz\text{-}f1^{RNAi2}$. Females with such genetic manipulation (named $ftz\text{-}f1^{RNAi}$ females) laid significantly fewer eggs than control females (Fig. 4.4D and Fig. 4.5D). In addition, females with $ftz\text{-}f1^{RNAi1}$ overexpression showed a severe retention of stage-14 follicles inside their ovaries (Fig. 4.5E), indicating an ovulation defect.

To support this observation, we examined whether stage-14 follicles from *ftz-f1*^{RNAi} females are competent to Octopamine (OA)-induced follicle rupture (Deady and Sun, 2015; Knapp et al., 2018). Using the *47A04-LexA* driving *LexAop2-6XGFP* as a reporter for isolating mature follicles, we found that mature follicles from control females had ~83% of follicles ruptured after OA stimulation, consistent with our previous result (Deady and Sun, 2015). In contrast, mature follicles from *ftz-f1*^{RNAi1} and *ftz-f1*^{RNAi2} females showed 6% and 17% follicle rupture rate, respectively (Fig. 4.5F). Since hexameric GFP showed punctate GFP signal in mature follicle cells (Fig. 4.5J-L), we also used *Oamb-RFP* as a reporter for isolating mature follicles from both control and *ftz-f1*^{RNAi} females to perform OA-induced follicle rupture. We observed 67% follicle rupture for control females, but 2% and 18% follicle rupture for *ftz-f1*^{RNAi1} and *ftz-f1*^{RNAi2} females, respectively (Fig. 4.4E and Fig. 4.5G-I). All the data suggest that transient expression of Ftz-f1 in follicle cells from stage 10B to stage 12 is required for follicle rupture and ovulation.

Our recent work has demonstrated that OA/Oamb signaling leads to calcium influx, which activates both Mmp2 and Nox to regulate follicle rupture (Deady and Sun, 2015; Li et al., 2018). To determine what is defective in follicles from *ftz-f1*^{RNAi} females, we first examined whether ionomycin, a Ca^{2+} ionophore, is sufficient to induce these follicles to rupture. Mature follicles from control females showed 75% follicle rupture with ionomycin stimulation; however, mature follicles from *ftz-f1*^{RNAi} females only showed ~3% follicle rupture (Fig. 4.4E). Similar results were also found when mature follicles were isolated according to *LexAop2-6XGFP* (Fig. 4.5F). The incompetency of ionomycin to induce follicles from *ftz-f1*^{RNAi} females to rupture indicates components

downstream of the calcium rise is defective in these follicles. Consistent with this, we found that Mmp2 expression in posterior follicle cells was completely disrupted in stage-14 follicles from *ftz-f1*^{RNAi} females (Fig. 4.4F-G). In addition, we found that these follicles were defective in OA-induced and ionomycin-induced superoxide production (Fig. 4.4J-K), indicating that Nox expression might also be disrupted in mature follicles of *ftz-f1*^{RNAi} females. Furthermore, we noticed that *Oamb-RFP* expression became patchy in mature follicles of *ftz-f1*^{RNAi} females when examined in higher magnification (Fig. 4.4H-I). Follicles from *ftz-f1*^{RNAi} females also exhibited morphological defects in overall shape and dorsal appendage formation (Fig. 4.5M-O). Altogether, these results indicate that transient expression of Ftz-f1 in stage-10B–12 follicle cells is essential for follicles to mature and become competent to OA-induced follicle rupture and ovulation.

Ftz-f1 promotes follicle cell differentiation into the final maturation stage

We have recently demonstrated that follicle cells experience a novel transition from stage 13 to stage 14 by downregulation of Cut and Ttk69 and upregulation of Hnt, which promotes Oamb and Mmp2 expression and follicle maturation for ovulatory competence (Knapp et al., 2019). Analysis of Hnt expression in stage-14 follicles from *ftz-f1*^{RNAi} females revealed a patchy expression of Hnt that overlaps with *Oamb-RFP* expression (Fig. 4.6A-B). In addition, Cut and Ttk69 were still detected in follicle cells without *Oamb-RFP*, consistent with the fact that Cut antagonizes Hnt expression in stage-14 follicle cells (Fig. 4.6C-F). The patchy nature of follicle cell markers is likely due to the incomplete knockdown of *ftz-f1* using RNAi. All these data support the

hypothesis that *ftz-f1* is required for follicle cells to transition into the final maturation stage.

To determine whether Ftz-f1 functions cell-autonomously in follicle cell differentiation, we utilized the MARCM system to generate *ftz-f1* mutant follicle-cell clones. Consistent with our hypothesis, *ftz-f1* mutant clones did not upregulate Hnt expression and continued to express Cut and Ttk69 in stage-14 follicles in a cell-autonomous fashion (Fig. 4.7A-C). In addition, EcR.A and EcR.B1, two isoforms downregulated in wild-type stage-14 follicle cells, were still detected at the high level in *ftz-f1* mutant follicle cells (Fig. 4.7D-E). Furthermore, we also found that another zinc-finger transcription factor Broad-Complex (Br-C; DiBello et al., 1991) was downregulated in neighboring wild-type follicle cells but remained high in *ftz-f1* mutant follicle cells (Fig. 4.7F). Finally, *ftz-f1* mutant follicle cells continue to have puncta of EDU staining, reflecting gene amplification, while neighboring wild-type follicle cells have already ceased gene amplification in stage 14 (Fig. 4.7G). All these data suggest that Ftz-f1 functions cell-autonomously to promote follicle cell differentiation into stage 14.

To determine which stages *ftz-f1* mutant follicle cells were arrested in, we carefully examined Hnt and Cut expression in *ftz-f1* mutant clones from stage 10B to stage 13. Previous work has shown that upregulation of Ttk69 in stage-10B follicle cells leads to downregulation of Hnt and upregulation of Cut (Sun et al., 2008), thus at the end of stage 10B, Hnt is undetectable, while Cut is fully upregulated. Indeed, we found that Hnt was downregulated in *ftz-f1* mutant clones at stage 10B; however, Hnt expression was not fully diminished in *ftz-f1* mutant clones at stage 10B or stage 12

(Fig. 4.7H-I). In addition, Cut expression was upregulated in *ftz-f1* mutant clones at stage 10B, but it was not upregulated as high as that in neighboring wild-type follicle cells (Fig. 4.7J). Such difference was undetectable at stage 12 when Cut is downregulated in wild-type follicle cells (Fig. 4.7K). Altogether, these data suggest that *ftz-f1* mutant follicle cells were arrested at the end of stage 10B. Therefore, ecdysone-induced Ftz-f1 expression is essential for follicle cell differentiation and progression into the final stages of maturation.

Sim is a downstream target of Ftz-f1 in stage-10B follicle cells

To understand how Ftz-f1 promotes follicle cell differentiation in late oogenesis, we focused on the basic helix-loop-helix (bHLH) transcription factor Single-minded (Sim), which was identified in an ongoing genetic screen for *Drosophila* ovulatory genes. Sim plays an essential role in embryonic neuronal development by regulating the proper differentiation of CNS midline cells (Crews, 1988; Nambu et al., 1991; Thomas et al., 1988). In addition, mutation of *sim* has shown to lead to oogenesis defects and female sterility with unknown mechanism (Pielage et al., 2002). In our results, antibody staining showed that Sim protein was not expressed in follicle cells before stage 10B, except in stalk follicle cells (Fig. 4.8A and Fig. 4.9). Sim was drastically upregulated in stage-10B/11 follicle cells (except stretch follicle cells in the anterior half of the egg chamber) and progressively downregulated to the lowest point at stage 13 (Fig. 4.8B-E). Sim was re-upregulated at stage 14 and its function at this stage will be reported in another manuscript (Fig. 4.8F). Upregulation of Sim at stage 10B prompted us to determine whether Ftz-f1 induces Sim expression. Consistent with this hypothesis, *ftz-f1*

mutant follicle cells completely lack Sim expression at stage 10B and 12 (Fig. 4.8G-H). In contrast, *ttk*-knockdown follicle cells have normal expression of Sim (Fig. 4.8I). These data suggest that Sim is a downstream target of Ftz-f1 but not Ttk69.

Sim functions downstream of Ftz-f1 to regulate follicle cell maturation

To determine whether Sim is required to regulate follicle cell differentiation, we generated flip-out clones with overexpression of sim^{RNAi}. Follicle cells with sim^{RNAi} overexpression have no detectable Sim expression at stage 10B, 12, or 14 (Fig. 4.3B-D), indicating efficient knockdown. Similar to the *ftz-f1* mutant follicle cells, sim^{RNAi}-overexpressing follicle cells also failed to fully upregulate Hnt expression at stage 14 (Fig. 4.10A), as well as downregulate Cut, Ttk69, EcR.A, EcR.B1, and Br-C (Fig. 4.10B-F). In addition, occasional faint expression of Hnt was detected in *sim*-knockdown follicle cells at stage 10B and 12 (Fig. 4.10G-H), while the difference of Cut expression in *sim*-knockdown and adjacent wild-type follicle cells was detected at stage 10B but not at stage 12 (Fig. 4.10I-J). The similarity between *ftz-f1* mutant and *sim*-knockdown follicle cells is not due to Sim regulating Ftz-f1 expression, as Ftz-f1 is properly upregulated in *sim*-knockdown follicle cells at stage 10B (Fig. 4.10K). All these data support the idea that Sim acts downstream of Ftz-f1 to promote follicle cell differentiation and maturation.

Mouse SF-1 is sufficient to replace Ftz-f1's role in follicle cell maturation

We aimed to rescue follicle cell differentiation defects in *ftz-f1* mutants with misexpression of *sim*. Unfortunately, ectopic *sim* expression in follicle cells during early oogenesis led to disrupted follicle cell organization and endoreplication reflected by its

smaller nuclei at stage 10A (Fig. 4.11A-B). These follicle cells can not properly transition into stage 10B, manifested by the continuous expression of Hnt and no upregulation of Cut after stage 10B (Fig. 4.11C-D).

Instead, we investigated the potential of mouse SF-1 (mSF-1), the mammalian homolog of Ftz-f1, to substitute for Ftz-f1 in follicle cell maturation. We generated follicle cell flip-out clones that express either *ftz-f1*^{RNAi2}, *mSF-1,* or both and examined follicle cell maturation markers. Consistent with the phenotype seen in *ftz-f1* mutant follicle cells (Fig. 4.7A), *ftz-f1*^{RNAi2}-overexpressing follicle cells could not upregulate Hnt expression at stage 14 (Fig. 4.12A). In contrast, follicle cells with both *ftz-f1*^{RNAi2} and *mSF-1* had normal Hnt upregulation at stage 14, the same as follicle cells with *mSF-1* alone (Fig. 4.12B-D). In addition, follicle cells with *ftz-f1*^{RNAi2} showed strong Cut expression at stage 14, while follicle cells with both *ftz-f1*^{RNAi2} and *mSF-1* had no Cut expression, similar to follicle cells with *mSF-1* alone (Fig. 4.12E-H). Altogether, these data suggest that mSF-1 can replace Ftz-f1's role in promoting follicle cell differentiation and maturation.

Strikingly, we also noticed that ectopic mSF-1 was sufficient to promote premature differentiation of follicle cells. In wild-type follicle cells, Hnt expression was not downregulated until stage 10B; however, Hnt was prematurely downregulated in follicle cells with both *mSF-1* and *ftz-f1*^{RNAi2} at stage 10A but not in earlier stages (Fig. 4.12I-J). In addition, Hnt was not re-upregulated until stage 14 in wild-type follicle cells but was prematurely upregulated in follicle cells with both *mSF-1* and *ftz-f1*^{RNAi2} at stages 12/13 (Fig. 412K-L). In accordance with Hnt, Cut was prematurely upregulated in follicle cells with both *mSF-1* and *ftz-f1*^{RNAi2} at stage 10A and prematurely

downregulated at stage 12/13 (Fig. 4.12M-P). It appeared that Cut was evicted from follicle cell nuclei for degradation (Fig. 4.12O-P). These data indicate that overexpression of *mSF-1* is sufficient to promote follicle cell differentiation prematurely and further support the notion that Ftz-f1 and mSF-1 play a conserved role in follicle cell differentiation and maturation.

Mouse SF-1 is sufficient to induce Sim expression in the absence of Ftz-f1

The rescue of follicle cell maturation by mSF-1 prompted us to examine whether mSF-1 is also sufficient to restore Sim expression in *ftz-f1*–knockdown follicle cells. Like *ftz-f1* mutant clones (Fig. 4.8G-H), Sim is barely detected in follicle cells with *ftz-f1*^{RNAi2} overexpression at stage 14; however, it is readily detected in follicle cells with both *ftz-f1*^{RNAi2} and *mSF-1* or *mSF-1* alone (Fig. 4.13A-D). Most strikingly, ectopic *mSF-1* was able to prematurely induce Sim expression in follicle cells with *ftz-f1*^{RNAi2} at stage 10A but not before stage 10A (Fig. 4.13E-F). In addition, Sim was also prematurely downregulated in these follicle cells at stage 12 (Fig. 4.13G-H). All these data are consistent with the idea that ectopic *mSF-1* promotes the premature differentiation of follicle cells via Sim. In conclusion, our data suggest that ecdysone-induced Ftz-f1 promotes follicle cell differentiation and maturation via bHLH transcription factor Sim, and this role is likely conserved (Fig. 4.13I).

Discussion

Ftz-f1 functions in adult ovaries to regulate female fertility in *Drosophila*

Work in this study demonstrated for the first time that Ftz-f1 is expressed in the adult ovarian follicle cells and is essential for female fertility. Since the characterization

of Ftz-f1 almost three decades ago (Lavorgna et al., 1991, 1993; Ueda et al., 1990), previous work has primarily focused on Ftz-f1's role in regulating early larval development, pupation, and metamorphosis (Broadus et al., 1999; Murata et al., 1996; Woodard et al., 1994; Yamada et al., 2000). These postembryonic developmental transitions required for metamorphosis into the adult fly are triggered through pulses of 20E (Riddiford, 1993), and Ftz-f1 is expressed downstream of the complex ecdysone-induced gene cascade, however only in periods between these pulses, with high levels of 20E shown to inhibit Ftz-f1 expression (Lavorgna et al., 1993; Richards, 1976; Sullivan and Thummel, 2003; Woodard et al., 1994). It is not fully understood what regulates Ftz-f1 expression during these periods, however some studies have revealed the ecdysone induced nuclear receptor DHR3 binds to cis-regulatory regions of the Ftz-f1 gene to induce high levels of Ftz-f1 expression during the mid-prepupal period and another ecdysone induced nuclear receptor E75B negatively regulates this induction through binding to DHR3 and acting as a co-repressor (Kageyama et al., 1997; Lam et al., 1997; White et al., 1997). In addition to these temporal regulators of Ftz-f1 expression, another nuclear receptor, DHR4, was also shown to be directly induced by 20E in third instar larvae, and is implicated to also be required for full Ftz-f1 expression (King-Jones et al., 2005). Our findings demonstrate that Ftz-f1 expression in the ovarian follicle cells is also induced downstream of ecdysone signaling, however it has yet to be determined if this is direct or indirect, and unlike development it seems Ftz-f1 is expressed at a time of active ecdysone signaling. Consistent with these differences, Ftz-f1 is also only expressed in follicle cells during one transient period of oogenesis

and does not present a cyclical pattern of expression with EcR signaling throughout the stages of oogenesis, unlike what is seen during development.

More insight into the relationship between Ftz-f1 and ecdysone signaling has emerged from recent work studying the role of Ftz-f1 in the fat bodies of the yellow fever mosquito, *Aedes aegypti*. These studies demonstrated in mosquitos Ftz-f1 interacts directly with a p160 steroid receptor coactivator FISC (Ftz-f1 Interacting Steroid receptor Co-activator), and Ftz-f1 is required to guide recruitment of the functional EcR/USP/FISC complex to 20E inducible target promoters (Zhu et al., 2006). Interestingly, FISC most closely resembles the *Drosophila* steroid hormone receptor co-activator Taiman, which has also shown to act as co-activator of ecdysone dependent transcription in the germarium and border cells of the ovary (Jang et al., 2009; König et al., 2011). Both Taiman and FISC belong to the family of bHLH-PAS transcription factors which require heterodimerization with other bHLH-PAS members to become active (Kewley et al., 2004; Zhu et al., 2006). Our findings show Ftz-f1 induces expression of Sim in the follicle cells, which is also characterized by bHLH-PAS domains, thus it will be interesting to investigate in future studies if any relationship exists between Taiman, Sim, and Ftz-f1 to regulate ecdysone-induced gene expression in these follicle cells.

Here we demonstrate Ftz-f1 signaling in follicle cells is critical for female fertility by regulating the ability of these follicle cells to properly mature into a state competent for follicle rupture and ovulation. Aside from Ftz-f1's role in regulating follicle cell maturation, it also seems that Ftz-f1 signaling in these follicle cells is required for dorsal appendage morphology, a complex process that requires proper cell patterning and

coordination of multiple signaling components (Boyle and Berg, 2009; Peters et al., 2013; Tzolovsky et al., 1999; Ward and Berg, 2005). Thus, it seems Ftz-f1 signaling in ovarian follicle cells may play multiple roles in follicles during late oogenesis, and it will be interesting to elucidate these additional mechanisms regulated by Ftz-f1 in the future.

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Ftz-f1 is required for follicle cell maturation in late oogenesis after stage 10B

Stage 10B of *Drosophila* oogenesis has already been identified as a critical transition point for follicle cells, as they switch out of endocycle from stage 10A into a phase of gene amplification at stage 10B (Calvi et al., 1998). This shift into gene amplification is executed through EcR activated upregulation of the transcription factor Ttk69 in stage-10B follicle cells (Sun et al., 2008). Our findings illustrate Ftz-f1 expression is also induced in follicle cells at stage 10B downstream of EcR signaling, however Ftz-f1 is not functioning in the previously identified Ttk69-GA pathway. Our findings demonstrate Ftz-f1 works in a pathway independent of the GA switch, and instead is required for follicle cells to mature past stage 11. Further work needs to be done to determine how Ftz-f1 is required for follicle cells to progress past stage 11, and understand the mechanism that is disrupted when loss of Ftz-f1 causes follicle cells to stay arrested in this stage. Recent work on the role of Ftz-f1 in the pupation transition has shown the tight temporally regulated shift from prepupa to pupa is mediated through Ftz-f1's induction of shade expression (Akagi et al., 2016). Even though shade is also expressed in the follicle cells during late oogenesis, expression of shade is not

regulated by Ftz-f1 (Fig. 4.14), suggesting the mechanism for Ftz-f1 mediated maturation in follicle cells differs from that in pupation.

In addition to inducing the switch to GA at stage 10B, earlier studies showed ecdysone signaling is crucial for follicles to progress past stage 10 through regulating a nutritional checkpoint and yolk uptake in coordination with the sesquiterpenoid juvenile hormone (JH) (Bownes, 1989; Carney and Bender, 2000; Soller et al., 1999; Terashima et al., 2005). The mechanism of JH signaling still remains unclear, however recent work has shown the bHLH-PAS protein Methoprene-tolerant (Met) can function as a receptor for JH, and furthermore binding of JH to Met causes conformational changes and allows Met to heterodimerize with Taiman (Charles et al., 2011). Furthermore, in-vitro studies indicate Ftz-f1 can form heterodimers with Met, and Ftz-f1 is crucial for JH induced gene expression (Dubrovsky et al., 2011) and recent work has also implicated Met is transiently expressed in follicle cells from stage 8 to 10 (Baumann et al., 2017). Altogether, it seems possible ecdysone signaling through Ftz-f1 could also be connected to JH signaling during these stages, and it will be important to untangle this intricate hormonal signaling pathway in future studies.

Another potential target to investigate stems from previous work demonstrating Ftz-f1 binds to an enhancer for the alcohol dehydrogenase gene (*Adh*) and can activate transcription (Ayer and Benyajati, 1992; Ayer et al., 1993a), ADH along with Aldehyde dehydrogenase (ALDH) are required for metabolizing ethanol into Acetyl-CoA, a key player in the Krebs cycle (Heinstra et al., 1983). Recent studies have found that loss of ALDH in females disrupts the ability of egg chambers to develop after stage 10 of oogenesis (Elgart et al., 2016). Thus, it would be interesting to investigate if Ftz-f1 is

required for metabolic regulation of follicle cells after stage 10 as a critical mechanism for follicle cell maturation.

The transcription factor Sim functions as a novel target of Ftz-f1

The function of Sim has been extensively studied in the development of the nervous system, as it is shown to regulate proper formation of the CNS midline, the central complex, and optic ganglia (Nambu et al., 1991; Pielage et al., 2002; Umetsu et al., 2006). Our findings here also illustrate for the first time that the bHLH-transcription factor Sim is expressed in ovarian follicle cells and functions as novel target of Ftz-f1 signaling to regulate follicle cell differentiation during late oogenesis. Our results also demonstrate that ectopic expression of Sim in follicle cells during earlier stages disrupted follicle cell organization and *sim*-expressing follicle cells bulged out abnormally toward the basal side away from the wildtype follicle cells. These abnormalities suggest Sim may play a role in regulating the expression of adhesion proteins in the follicular epithelium, consistent with this possibility previous work has also shown Sim mediated axon fasciculation in the larval brain may be regulated through the proper expression of adhesion proteins (Freer et al., 2011).

Our findings also show expression of Sim is re-upregulated in stage-14 follicle, suggesting it might serve another function in preovulatory follicles. Thus, additional work will need to be done to elucidate the function of Sim in this final stage of folliculogenesis.

Conservation of NR5A nuclear hormone signaling in ovarian follicle cells

The mammalian NR5A homolog SF-1, is expressed in somatic follicle cells of the ovary in both rodents and humans (Hinshelwood et al., 2003; Tajima et al., 2003), and loss of this SF-1 expression in murine granulosa cells leads to a severe depletion of developing follicles and infertility (Pelusi et al., 2008b). Despite the critical role for SF-1 in female fertility, it still remains unknown how SF-1 within these follicle cells regulates folliculogenesis.

Drosophila poses as a valuable model to further study the function of NR5A receptors, considering the DNA binding sequence of NR5A receptors is highly conserved from *Drosophila* to humans, with over 80% in sequence similarity (Fayard et al., 2004). Furthermore, studies have already begun to show the functional conservation of these NR5A receptors in both the embryo and female reproductive tract of Drosophila (Lu et al., 2013; Sun and Spradling, 2012). In our work we demonstrate that Ftz-f1 is also expressed in the somatic follicle cells of the ovary and plays a crucial role in female fertility, akin to SF-1. Furthermore, our work demonstrates that Ftz-f1's function in follicle cell maturation is functionally conserved, as mSF-1 is sufficient to rescue defects in follicle cell differentiation. Our results also show SF-1 is sufficient to induce expression of the Ftz-f1 signaling target Sim, thus it would be interesting to probe if in mammalian follicle cells the homolog of Sim (Yamaki et al., 1996) is also expressed, and if it indeed functions downstream of SF-1. Overall our findings could help to further elucidate the genetic and molecular mechanisms of NR5A signaling and how it regulates follicle development and female fertility.

Materials & Methods

Drosophila genetics

Flies were reared on standard cornmeal and molasses food at 25°C and experiments performed at 29°C, unless noted otherwise. *ftz-f1^{ex7}, FRT 79D* was obtained from Jean Maurice Dura. Additional expression patterns of Ftz-f1 (aside from Ftz-f1 antibody immunostaining) utilized *ftz-f1-GFP.FLAG* (Bloomington *Drosophila* Stock Center (BDSC), stock #38645) and ftz-f1^{2877}/TM3 (Karpen and Spradling, 1992). The *Vm26Aa-Gal4* (Peters et al., 2013) was used to drive expression in main-body follicle cells starting at stage-10. Isolation and identification of stage-14 follicles were performed using *Oamb-RFP* (Knapp et al., 2019) or *47A04-LexA* (BDSC, stock #54873) to drive *lexAop2-GFP* (BDSC, stock #52265). The protein trap line *Mmp2::GFP* (Deady et al., 2015) was used for Mmp2 expression. Control flies for all experiments were prepared by crossing Gal4 driver to Oregon-R flies. The following transgenic lines were used to knock down or overexpress genes in experiments: *UAS-EcR^{DN}* (BDSC, stock #6872), *UAS-ttk^{RNAi}* (Vienna *Drosophila* Resource Center (VDRC), stock #10855), UAS-*ftz-f1^{RNAi1}* (BDSC, stock #33625), *UAS- ftz-f1^{RNAi2}* (VDRC, stock #104463), *UAS-sim^{RNAi}* (VDRC, stock #26888), *UAS-sim-3xHA* (Fly-ORF, stock #000719) and *UAS-mSF1* (Yussa et al., 2001). The following marker lines were used for clonal misexpression: *hsFLP;act<CD2<Gal4,UAS-GFP/TM3* and *hsFLP, tubGal4, UAS-GFP; tubGal80, FRT2A/TM6B.*

Generation of follicle cell clones

Mosaic analysis with repressible cell marker (MARCM) was used to generate follicle cell clones homozygous for the *ftz-f1*^{ex7} allele. Adult females were heat shocked for 45 minutes at 37°C to induce FLP/FRT mediated recombination and incubated at 25°C for 2-4 days for generation of *ftz-f1*-null follicle cell clones. For generation of flip-out clones, adult females were heat shocked for 50 minutes at 37°C and incubated at 25°C for 2-4 days before dissection.

Ovulation assays

Egg laying experiments were performed as previously described (Deady and Sun, 2015). Five-day-old females (fed with wet yeast for 1 day) were housed with Oregon-R males (5 females :10 males) in one bottle to lay eggs on molasses plates over two days at 29°C (with removal and replacement of plates every 22 hours). After egg laying ovary pairs for each female were dissected out and the number of mature follicles within were quantified.

The *ex vivo* follicle rupture assays were performed as described previously (Knapp et al., 2018). Ovaries from 5-6-day-old virgin females fed with wet yeast for 3 days were dissected out and stage-14 follicles were isolated in Grace's insect medium (Caisson Labs, Smithfield, UT, USA). After isolation, follicles were separated into groups ~30 follicles and cultured at 29°C for 3 hours in culture medium containing 20 μM OA (Sigma-Aldrich) or 2 μM ionomycin (Cayman Chemical, Ann Arbor, MI, USA). Each data point represents the percentage (mean ± standard deviation (SD)) of ruptured follicles per experimental group.

Superoxide detection

Measurement of superoxide production was performed as previously described (Li et al., 2018), with slight modifications. Mature follicles (5) were isolated and placed in each well of a 96-well plate with 600 µl of Grace's insect medium containing either 20 µM OA or 2 µM ionomycin and 200 µM of L-012 (Wako Chemicals). Plates were placed in a CLARIOstar microplate reader (BMG Labtech) for a 60 minute L-012 luminescence reading. Eight to ten wells (technical repeats) were used in each experiment for each genotype, and the mean ± standard error of the mean (SEM) of the technical repeats was calculated. Each experiment was performed at least twice.

Immunostaining, EdU detection, and microscopy

Immunostaining was performed following a standard procedure including ovary dissection, fixation in 4% EM-grade paraformaldehyde for 13 min, blocking in PBTG (PBS plus 0.2% Triton, 0.5% BSA and 2% normal goat serum), and primary and secondary antibody staining diluted in PBTG. The following primary antibodies were used: mouse anti-Hnt (1G9, 1:75), mouse anti-Cut (2B10, 1:15), mouse anti-BrC (25E9.D7, 1:15), mouse anti-EcR.A (15G1a, 1:30), and mouse anti-EcR.B1 (AD4.4, 1:30) from the Developmental Study Hybridoma Bank; rabbit anti-Ftz-f1 (1:100), rabbit anti-Ttk69 (1:100; a gift from Dr. Wanzhong Ge, Zhejiang University, China), rabbit anti-GFP (1:4000; Invitrogen), mouse anti-GFP (1:4000; Invitrogen), rabbit anti-RFP (1:2000, MBL international), Chicken anti-β-Gal (ab9361, 1:100; Abcam), guinea pig anti-Sim (1:100; a gift from Dr. Stephen Crews, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, USA). Alexa Fluor 488 and Alexa Flour 568 goat secondary antibody (1:1000; Invitrogen) were used as secondary antibodies.

EdU detection was performed as previously described (Alexander et al., 2015). Ovaries were dissected out in room temperature Grace's insect medium and incubated in 50 μM EdU for 30 minutes. Ovaries were then fixed in 4% EM-grade paraformaldehyde for 13 min and permeabilized in PBX (0.1% TritonX in PBS) for 30 mins. For detection of EdU the Invitrogen's Click-iT EdU Alexa Fluor 555 Imaging Kit was utilized following the manufacturer's instructions.

Images were acquired using a Leica TCS SP8 confocal microscope or Leica MZ10F fluorescent
stereoscope with a sCOMS camera (PCO.Edge) and assembled using Photoshop software (Adobe) and ImageJ.

Statistical analysis

Statistical tests were performed using Prism 7 (GraphPad, San Diego, CA). Quantification results are presented as mean±SD or mean±SEM as indicated. Statistical analysis was conducted using Student's **t**-test.

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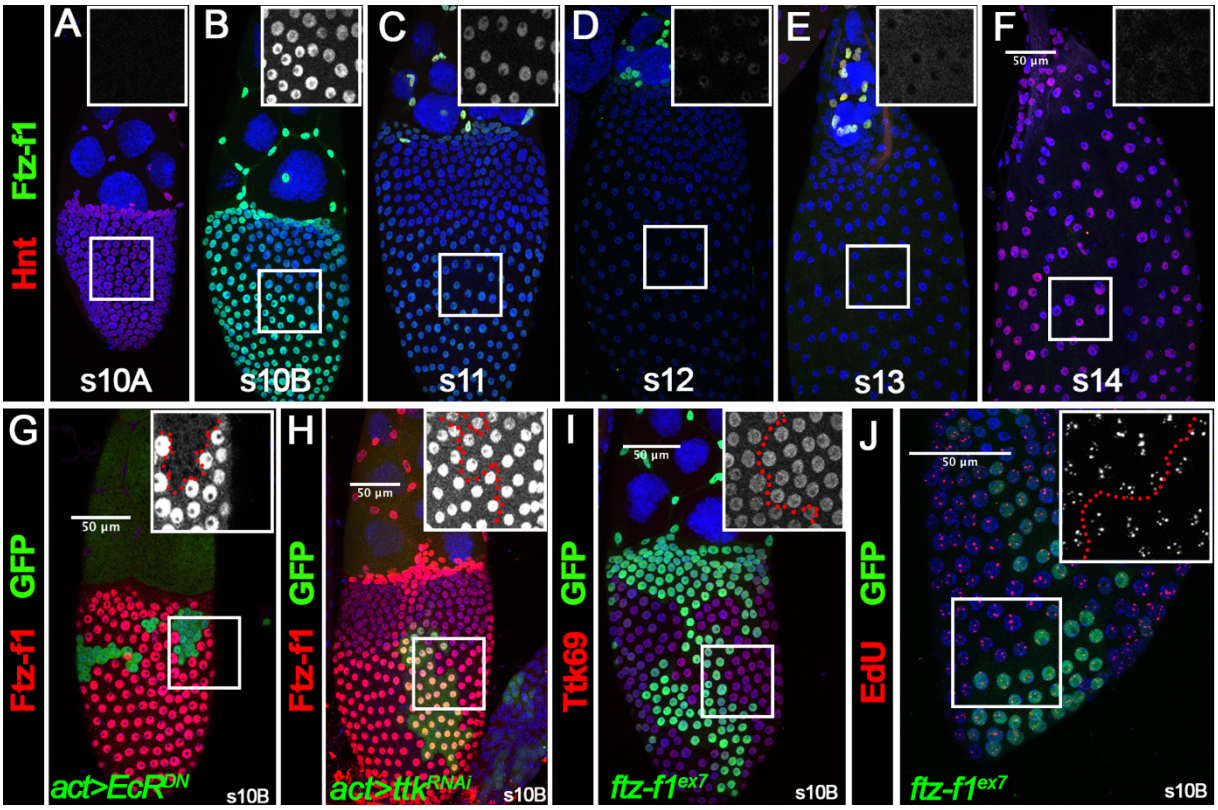

Figure 4.1. Ftz-f1 is downstream of EcR but independent of the Ttk69-GA switch in stage-10B follicle cells.

(A-F) Ftz-f1 is expressed in follicle cells during late oogenesis. Ftz-f1 protein (green) is first detected in follicle cells at stage-10B of oogenesis (B), and expression in main-body follicle cells decreases until stage-12 (C-D) and is undetectable in stage-13(E). Stage-10A (A) and stage-14 follicles (F) are marked by expression of Hnt (red). The insets are higher magnification of Ftz-f1 expression (white) in outlined areas. (G-J) Ftz-f1 functions downstream of EcR but independent of the Ttk69-GA switch at stage-10B. (G) Ftz-f1 (red, (white in inset)) was not expressed in follicle cell clones expressing EcR^{DN} (marked with GFP (green) and outlined in inset). (H) Expression of Ftz-f1 (red in H, (white in inset)) is not disrupted in follicle cell clones expressing ttk^{RNAi} (marked with GFP (green) and outlined in inset). (I-J) $ftz\text{-}f1^{ex7}$ mutant clones (marked with GFP (green) and outlined in insets) exhibit normal expression of Ttk69 (red in I, (white in inset)) and a punctate EdU incorporation pattern (red in J, (white in inset)). Insets show higher magnification of outlined areas. Nuclei are marked by DAPI in blue.

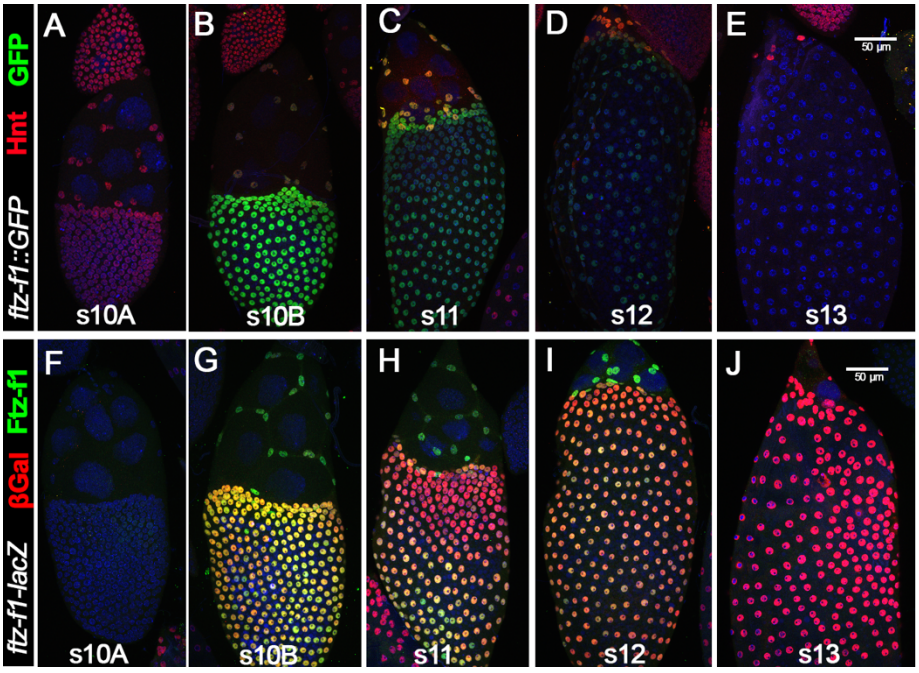

Figure 4.2. Expression pattern of Ftz-f1 in late oogenesis.

(A-E) Representative images show Ftz-f1::GFP expression (green) in oogenesis. Ftz-f1::GFP is first detected in stage-10B follicles (B), and decreases in expression until stage-12 (C-D), until it is completely lost by stage-13 (E). Hnt (red) marks stage-10A follicles (A). (F-J) Representative images show expression of *Ftz-f1-lacZ* detected via β-gal (red) and Ftz-f1 protein (green) in late oogenesis. Expression of both β-gal and Ftz-f1 protein begin in stage-10B follicles (G), but as Ftz-f1 protein decreases by stage-13, β-gal expression stays consistently robust (H-J). Nuclei are marked by DAPI in blue.

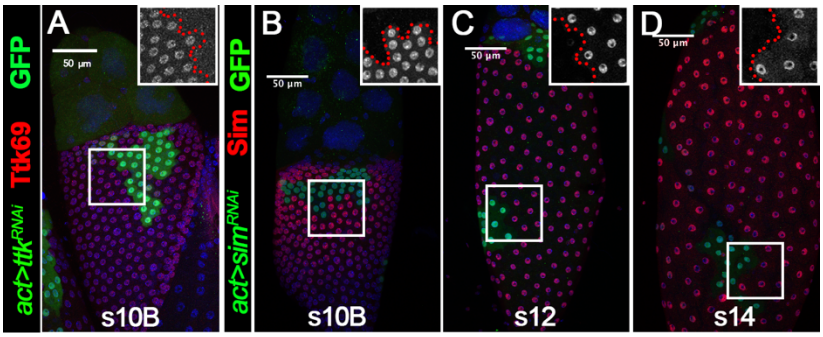

Figure 4.3. Expression of Ttk69 and Sim are efficiently knocked down by ttk^{RNAi} and sim^{RNAi} respectively.

(A) Expression of Ttk69 protein (red in A, (white in inset)) is knocked down in follicle cell clones expressing *ttk*^{RNAi} (marked with GFP (green) and outlined in inset). The insets show higher magnification of Ttk69 expression (white) in outlined areas. (B-D) Follicle cell clones expressing *sim*^{RNAi} (marked with GFP (green) and outlined in insets) efficiently knock down expression of Sim (red, (white in insets)) through late oogenesis. The insets are higher magnification of Sim expression (white) in outlined areas. Nuclei are marked by DAPI in blue.

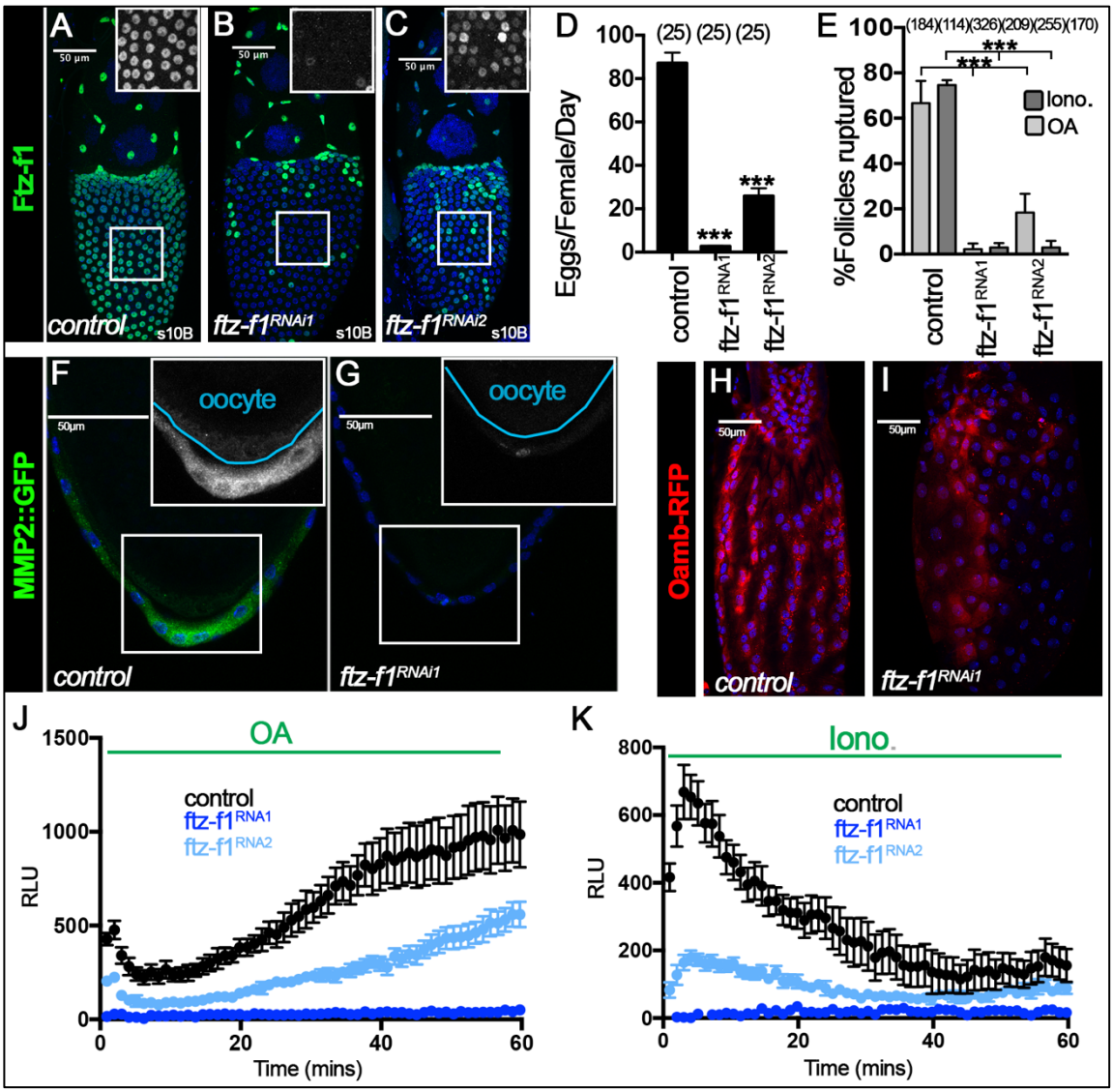

Figure 4.2. Ftz-f1 is required for follicle rupture and ovulation.

(A-C) Representative images show Ftz-f1 protein (green) in control (A) and *ftz-f1*^{RNAi} (B-C) stage-10B follicles driven by *Vm26Aa-Gal4*. The insets are higher magnification of Ftz-f1 expression (white) in outlined areas. (D) Quantification of egg laying in control or *ftz-f1*^{RNAi} females with *Vm26Aa-Gal4*. The number of females is noted above each bar. (E) Quantification of OA-induced (light grey bars) and Ionomycin-induced (dark grey bars) follicle rupture in control or *ftz-f1*^{RNAi} females with *Vm26Aa-Gal4*. Mature follicles were isolated according to *Oamb-RFP* expression. The number of mature follicles analyzed is noted above each bar. (F-G) Representative images show Mmp2::GFP expression (green) in control (F) or *ftz-f1*$^{RNAi\ 1}$ (G) mature follicles with *Vm26Aa-Gal4*. Insets show higher magnification of Mmp2::GFP expression (white) in outlined areas, oocytes are outlined in cyan. (H-I) Representative images show Oamb-RFP expression (red) in control (H) and *ftz-f1*$^{RNAi\ 1}$ (I) mature follicles with *Vm26Aa-Gal4*. (J-K) Quantification of L-012 luminescent signal (indicating superoxide production) in control (black), *ftz-f1*^{RNAi1}(dark blue), and *ftz-f1*^{RNAi2} (light blue) mature follicles with *VM26Aa-Gal4* from either OA (J) or Ionomycin (K) stimulation. Nuclei are marked by DAPI in blue. ***P<0.001 (Student's t-test).

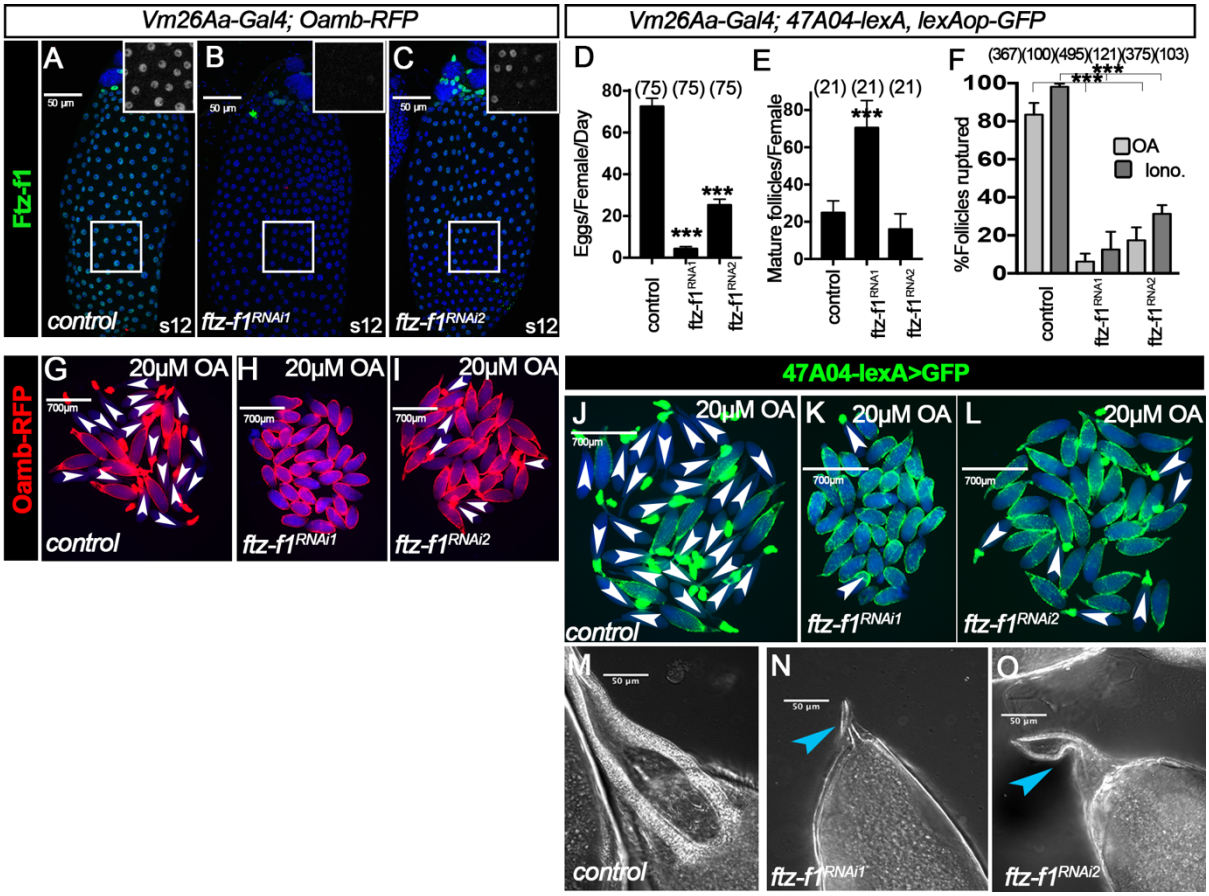

Figure 4.3. Knock down of Ftz-f1 causes defects in egg retention and morphology.

(A-C) Representative images show Ftz-f1 expression (green) in control (A) and *ftz-f1*^{RNAi} (B-C) follicles driven by *Vm26Aa-Gal4* is almost completely knocked down by stage-12. The insets are higher magnification of Ftz-f1 expression (white) in outlined areas. Nuclei are marked by DAPI in blue. (D-E) Quantification of egg laying (D) and mature follicles in each female's ovaries post egg laying (E) in control or *ftz-f1*^{RNAi} females with *Vm26Aa-Gal4; 47A04-lexA>lexAop-GFP.* The number of females is noted above each bar. (F) Quantification of OA-induced (light grey) or Ionomycin-induced (dark grey) follicle rupture in control or *ftz-f1*^{RNAi} females with *Vm26Aa-Gal4*. The mature follicles were isolated according to *47A04-lexA>lexAop-GFP* expression and the number of mature follicles analyzed is noted above each bar. (G-I) Representative images show control (G), *ftz-f1*^{RNA1i} (H), and *ftz-f1*^{RNAi2} (I) mature follicles with *VM26Aa-Gal4* after 3 h culture with OA. Mature follicles were isolated according to *Oamb-RFP* expression (red). Ruptured follicles are marked by arrowheads. (J-L) Representative images show control (J), *ftz-f1*^{RNA1i} (K), and *ftz-f1*^{RNAi2} (L) mature follicles with *VM26Aa-Gal4* after 3 h culture with OA. Mature follicles were isolated according to *47A04-lexA>lexAop-GFP* expression (green). Ruptured follicles are marked by arrowheads. (M-O) Representative DIC images show dorsal appendage morphology in control (M), *ftz-f1*^{RNA1i} (N), and *ftz-f1*^{RNAi2} (O) stage-14 follicles with *VM26Aa-Gal4.* Blue arrow-heads indicate stunted dorsal appendage formation. p-value <0.001 (***).

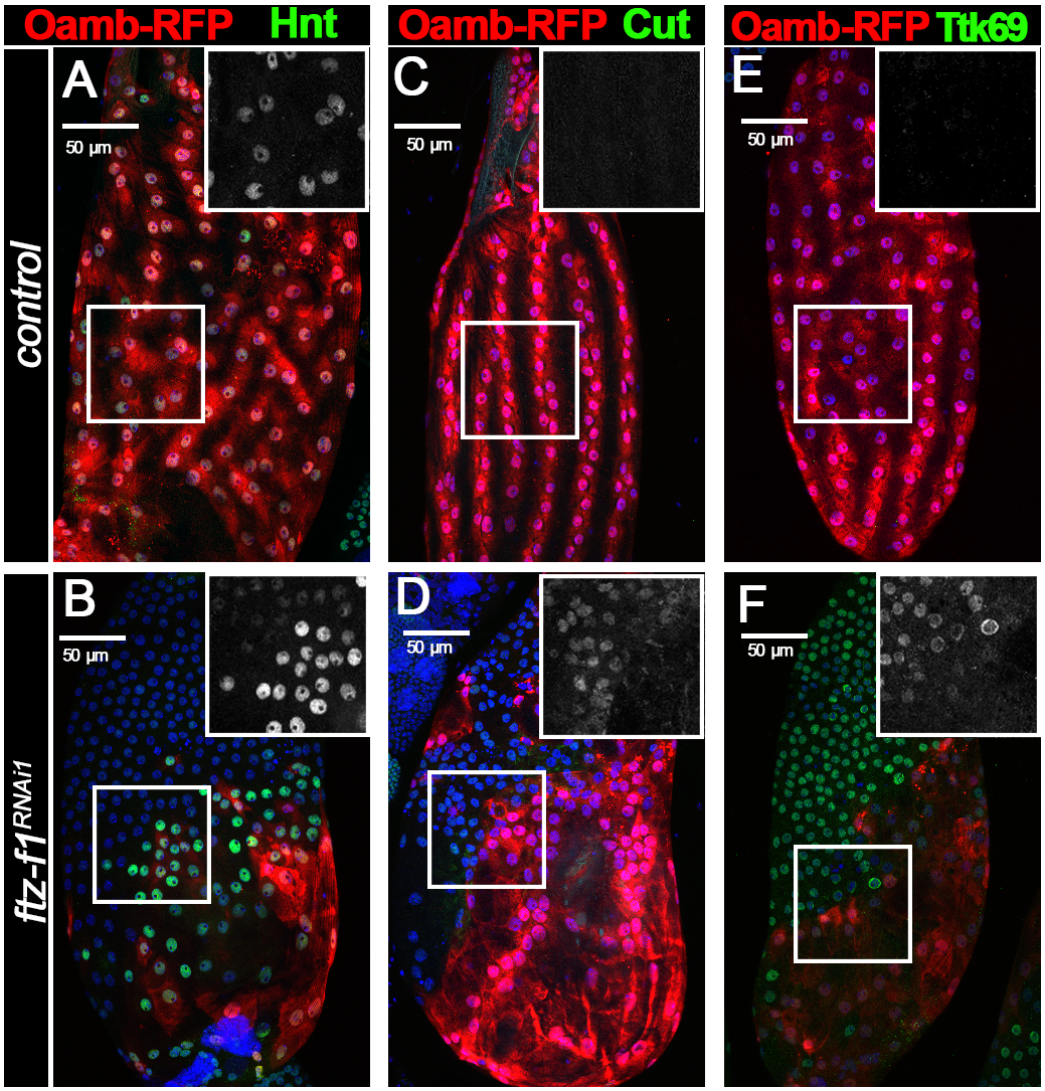

Figure 4.4. Knock down of Ftz-f1 in follicle cells disrupts the third follicle cell transition into stage 14.

(A-F) Representative images show Hnt expression (green in A-B and white in insets), Cut expression (green in C-D and white in insets), and Ttk69 expression (green in E-F and white in insets) in control (A,C,E) and *ftz-f1*^{RNAi1} (B,D,F) follicles driven by *Vm26Aa-Gal4*. The stage-14 follicle cells are marked by Oamb-RFP (red). The insets are higher magnification of antibody expression (white) in outlined areas. Nuclei are marked by DAPI in blue.

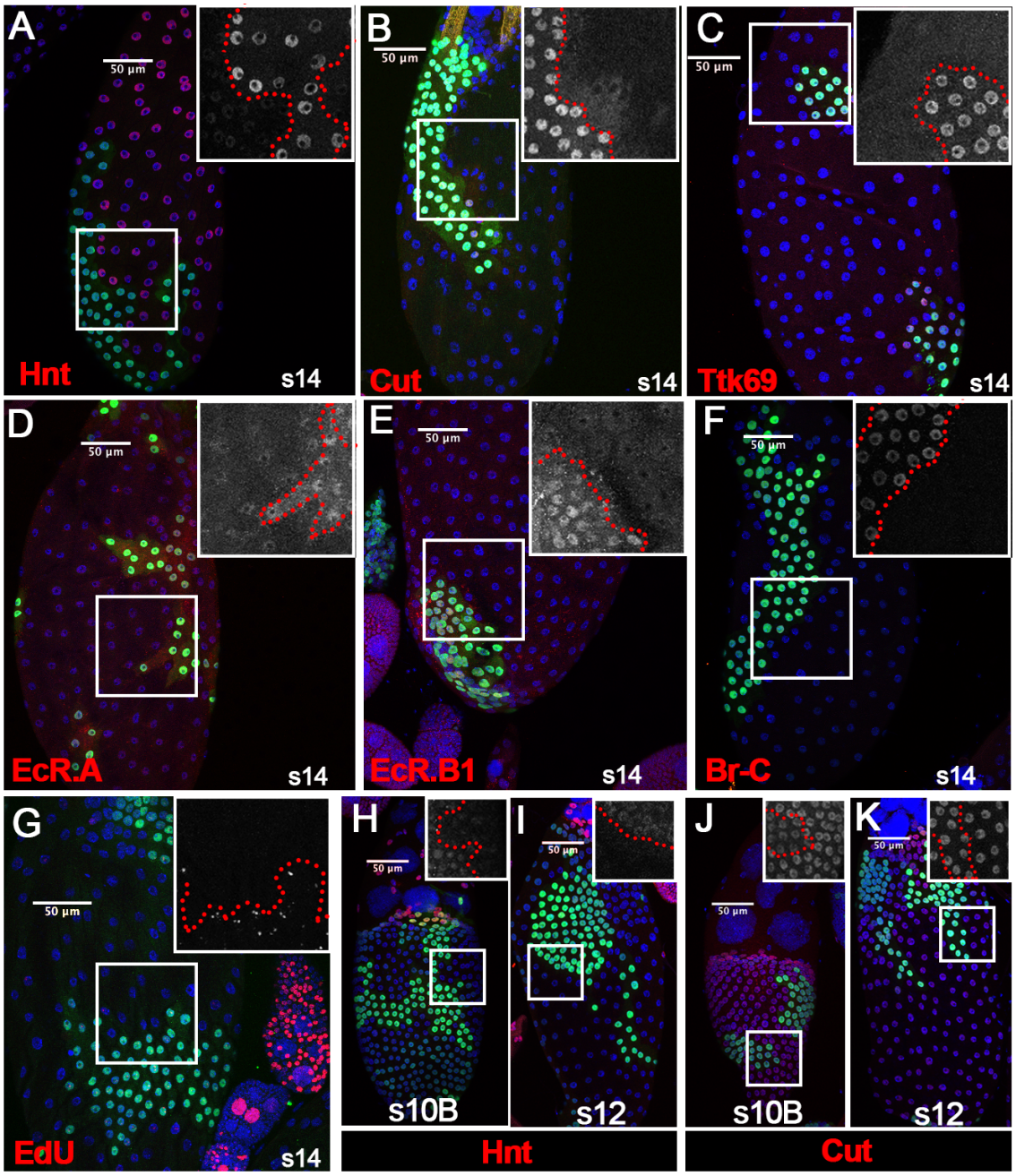

Figure 4.5. Ftz-f1 promotes follicle cell differentiation into the final maturation stage.

(A-K) *ftz-f1*^{ex7} mutant clones are marked by GFP (green) and outlined in higher magnified insets. (A-G) Representative images show *ftz-f1*^{ex7} mutant clones are unable to upregulate expression of Hnt (red in A, (white in inset)) and downregulate expression of Cut (red in B, (white in inset)), Ttk69 (red in C, (white in inset)), EcR.A (red in D, (white in inset)), EcR.B1 (red in E, (white in inset)), Br-C (red in F, (white in inset)), and EdU incorporation (red in G (white in inset)) in stage-14 follicles. (H-I) Expression of Hnt (red, (white in insets)) remains at very low levels in stage-10B (H) and stage-12 (I) follicles. (J-K) Moderate levels of Cut expression (red, (white in insets)) are seen in stage-10B (J) and stage-12 (K) follicles. Insets show higher magnification of outlined areas. Nuclei are marked by DAPI in blue.

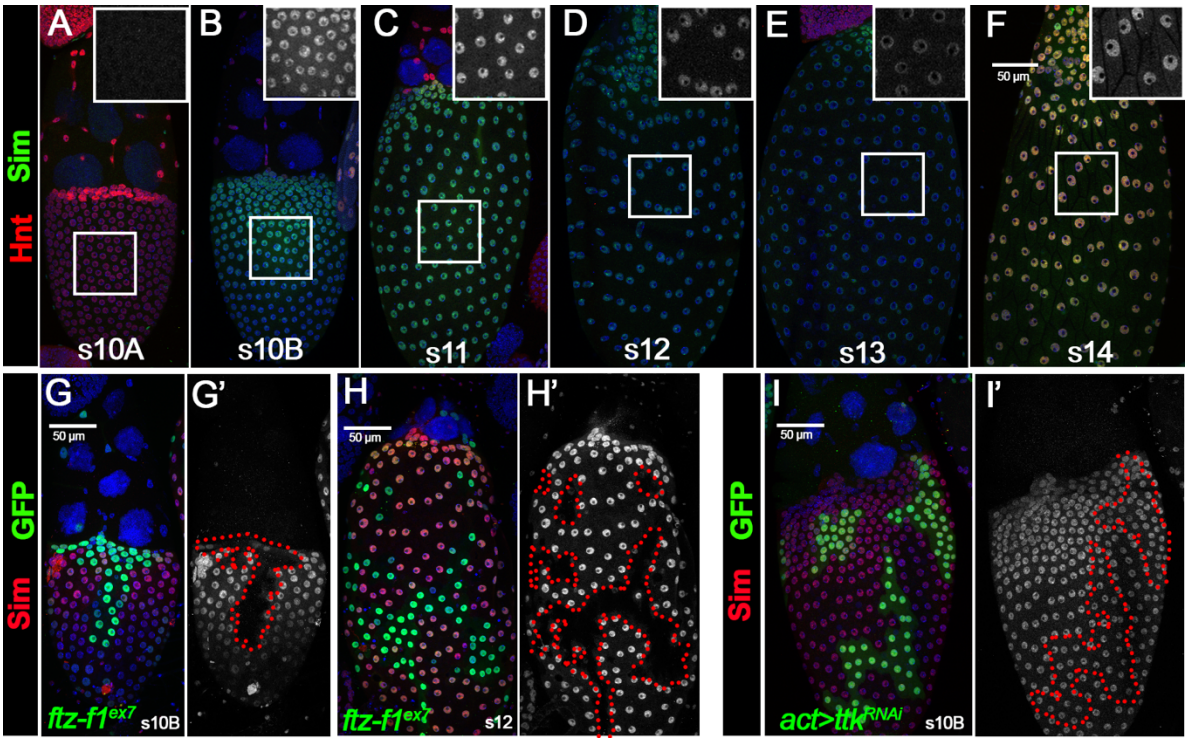

Figure 4.6.Ftz-f1 promotes expression of Sim in stage-10B follicle cells.

(A-F) Sim is expressed in follicle cells during late oogenesis. Sim protein (green) is expressed in main-body follicle cells starting at stage-10B (B), and downregulates from stage-11 to 13 (C-E), until stage-14 when expression is again enriched (F). Stage-10A (A) and stage-14 (F) follicles are marked by expression of Hnt (red). The insets are higher magnification of Sim expression (white) in outlined areas. (G-H) Expression of Sim (red in G-H and white in G',H') is lost in *ftzf1*^{ex7} mutant clones (marked with GFP (green in G,H) and outlined in G',H') in stage-10B follicles (G) and after (H). (I) Sim expression (red in I and white in I') is not affected in follicle cell clones expressing *ttk*^{RNAi} (marked with GFP expression (green in I) and outlined in I'). Nuclei are marked by DAPI in blue.

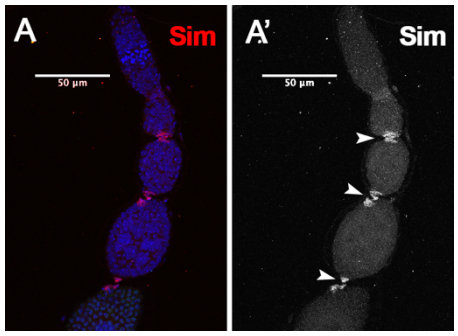

Figure 4.7. Stalk cells express Sim.

(A) Representative image shows Sim expression (red in A, white in A') in early stages of oogenesis. Nuclei are marked by DAPI in blue.

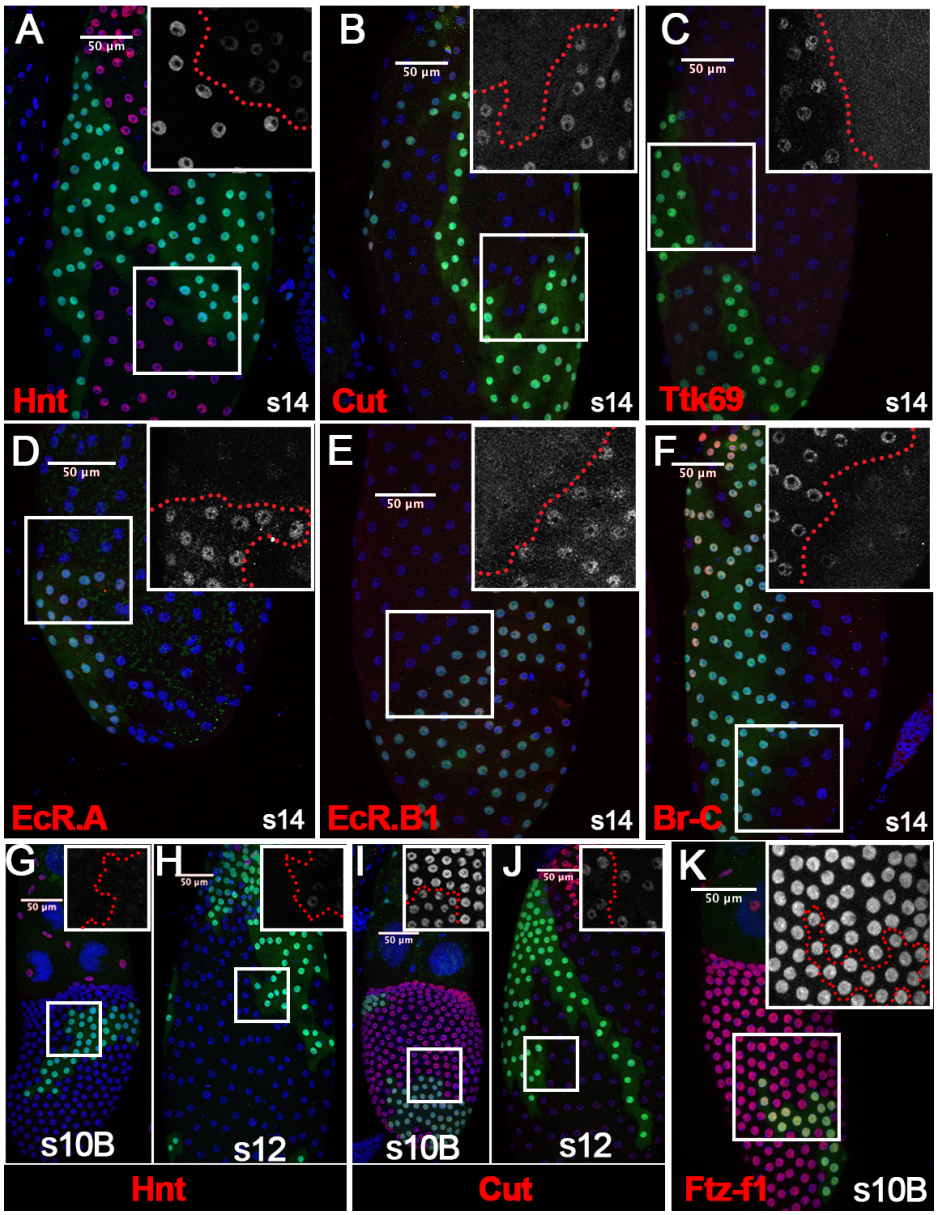

Figure 4.8.Sim promotes follicle cell differentiation into the final maturation stage.

(A-K) Follicle cell clones expressing sim^{RNAi} are marked with GFP (green) and outlined in higher magnified insets. (A-F) Representative images show follicle cells expressing sim^{RNAi} are unable to upregulate expression of Hnt (red in A, (white in inset)) and downregulate expression of Cut (red in B, (white in inset)), Ttk69 (red in C, (white in inset)), EcR.A (red in D, (white in inset)), EcR.B1 (red in E, (white in inset)), and Br-C (red in F, (white in inset)) in stage-14 follicles. (G-H) Expression of Hnt (red, (white in insets)) remains very low in stage-10B (G) and stage-12 (H) follicles. (I-J) Wildtype expression of Cut (red, (white in insets)) is seen in stage-10B (I) and stage-12 (J) follicles. (K) Expression of Ftz-f1 (red, (white in inset) in stage-10B follicles is not affected by sim knockdown. Insets show higher magnification of outlined areas. Nuclei are marked by DAPI in blue.

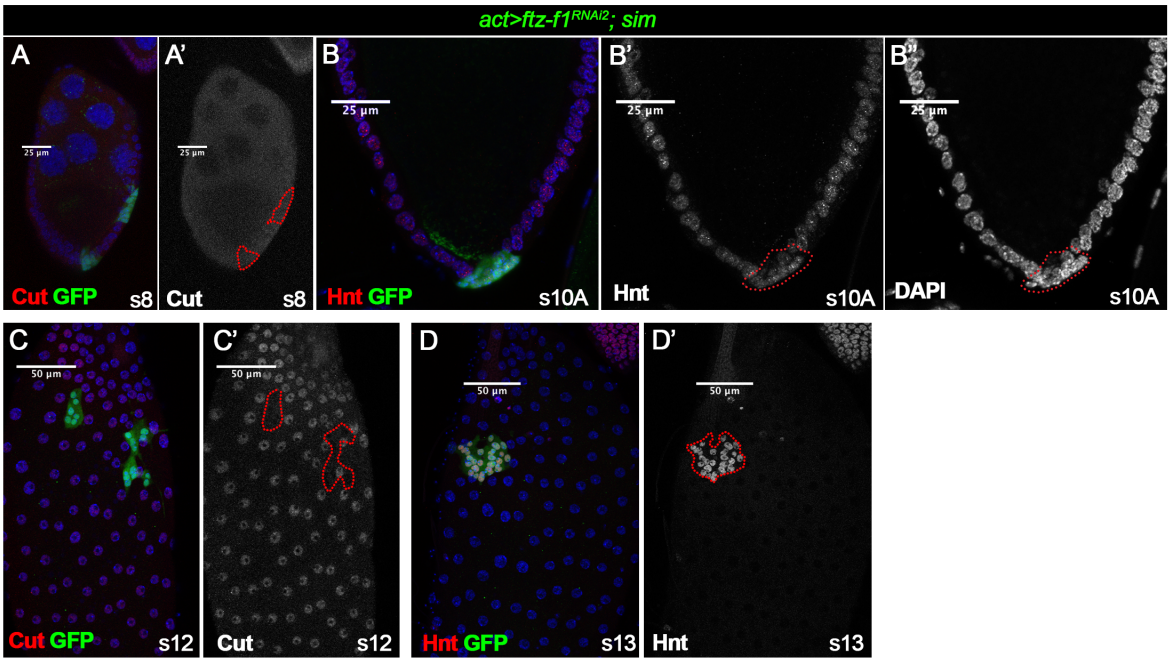

Figure 4.9. Ectopic expression of Sim in early stages of oogenesis disrupts earlier follicle cell transitions.

(A-D) Follicle cell clones expressing *ftz-f1*^{RNAi2}*;sim* (marked with GFP expression (green) and outlined) are unable to transition out of endocycle. (A) Follicle cells with ectopic *sim* are able to transition properly from the mitotic cycle into endocycle as indicated by downregulation of Cut expression (red in A and white in A') in stage-8 follicles. (B) In stage 10A expression of Hnt (red in B, and white in B') is not disrupted in *ftz-f1*^{RNAi2}*;sim* follicle cell clones, but DAPI staining (blue in B, and white in B'') shows abnormal bulging phenotype. (C-D) *ftz-f1*^{RNAi2}*;sim* follicle cells fail to transition out of endocycle, and do not upregulate expression of Cut (red in C and white in C') in stage-12 follicles and do not downregulate expression of Hnt (red in D and white in D') in stage-13 follicles. Nuclei are marked by DAPI in blue.

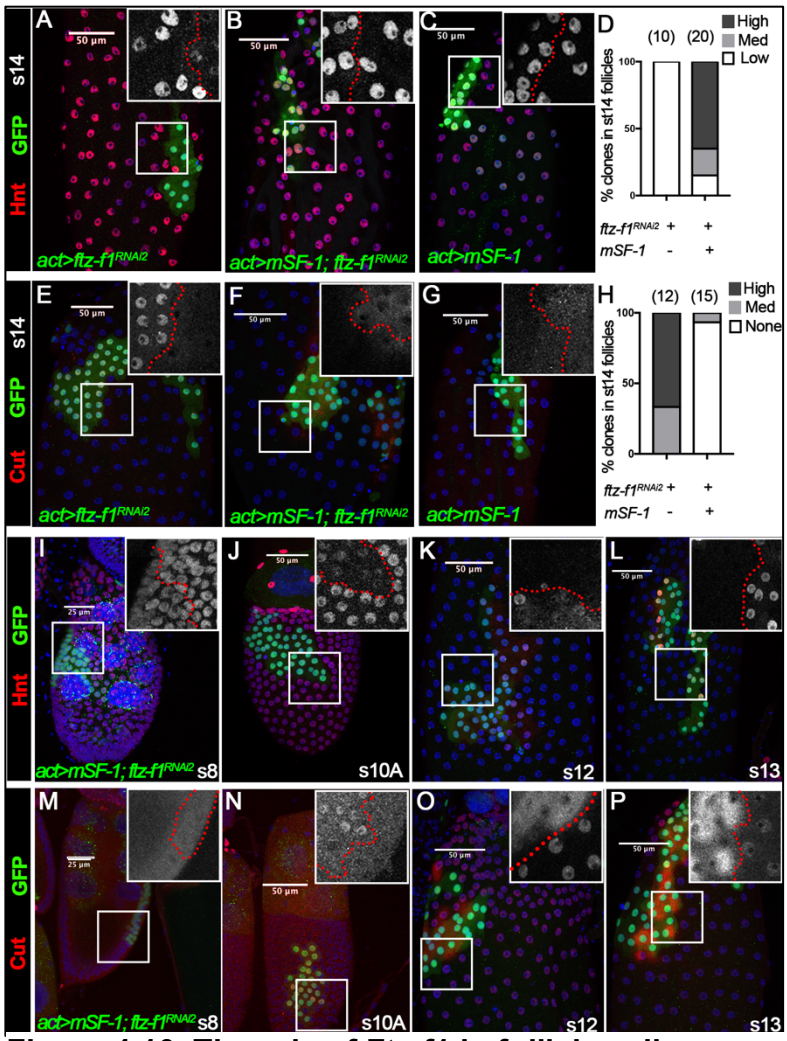

Figure 4.10. The role of Ftz-f1 in follicle cell maturation can be replaced by mSF-1.

(A,E) Follicle cell clones expressing *ftz-f1*^{RNAi2} (marked with GFP (green) and outlined in higher magnified insets) are unable to upregulate Hnt (red in A, (white in inset)) and downregulate Cut (red in E, (white in inset)). (B,C,F,G) Follicle cell clones expressing *mSF-1;ftz-f1*^{RNAi2} (B,F) or just *mSF-1* (C, G) (marked with GFP (green) and outlined in higher magnified insets) exhibit wildtype expression of Hnt (B-C) and Cut (F-G) (red, (white in insets)). (D,H) Quantification of Hnt (D) and Cut (H) expression in follicle cells expressing *ftz-f1*^{RNAi2} or *ftz-f1*^{RNAi2}*;mSF-1* in stage-14 follicles. The number of clones analyzed is noted above each bar. (I-P) Expression of *mSF-1;ftz-f1*^{RNAi2} pushes follicle cells to mature early starting at stage 10A. (I-L) Hnt expression (red, (white in insets)) in *mSF-1;ftz-f1*^{RNAi2} follicle cells is not affected in stage-8 (I), but starts to downregulate prematurely in stage-10A follicles (J). Hnt then begins to prematurely upregulate in stage-12 follicles (K), and is robustly expressed in stage-13 follicles (L). (M-P) Cut expression (red, (white in insets)) in *mSF-1;ftz-f1*^{RNAi2} follicle cells is also not affected earlier in stage-8 (M), but starts to prematurely upregulate in stage-10A follicles (N). Cut then begins to prematurely downregulate nuclear expression in stage-12 (O) and stage-13 (P) follicles. Nuclei are marked by DAPI in blue.

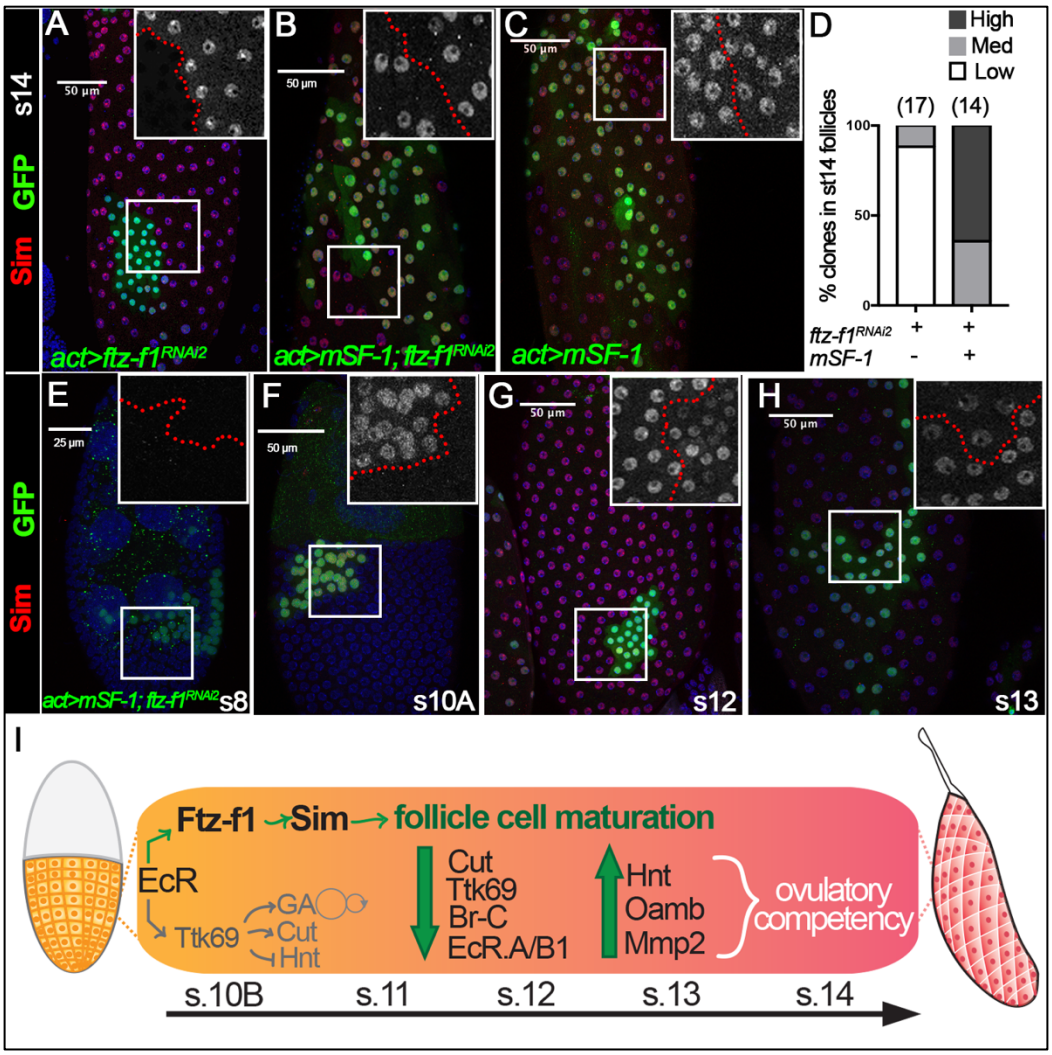

Figure 4.11. Sim expression can be rescued by mSF-1.

(A-D) Expression of mSF-1 can restore Sim expression when Ftz-f1 is disrupted. (A) Follicle cell clones expressing *ftz-f1*^{RNAi2} (marked with GFP expression (green) and outlined in higher magnified insets) fail to express Sim (red, (white in inset)) in stage-14 follicles. (B-C) Follicle cell clones expressing *mSF-1;ftz-f1*^{RNAi2} (B) or just *mSF-1* (C) (marked with GFP (green) and outlined in higher magnified insets) exhibit wildtype expression of Sim (red, (white in insets)) in stage-14 follicles. (D) Quantification of Sim expression in follicle cells expressing *ftz-f1*^{RNAi2} or *ftz-f1*^{RNAi2}*;mSF-1* in stage-14 follicles. The number of clones analyzed is noted above each bar. (E-H) Expression of *mSF-1;ftz-f1*^{RNAi2} (marked with GFP (green) and outlined in higher magnified insets) is sufficient to induce Sim expression prematurely at stage-10A (F) but not earlier (E), and is able to restore Sim expression though out late oogenesis (G-H). (I) Summary schematic of follicle cell maturation in late oogenesis. Expression of Ftz-f1 is induced downstream of EcR signaling in stage-10B follicles, independent of the Ttk69-GA switch, and promotes expression of Sim to regulate the maturation of follicle cells into a stage-14 preovulatory competent state.

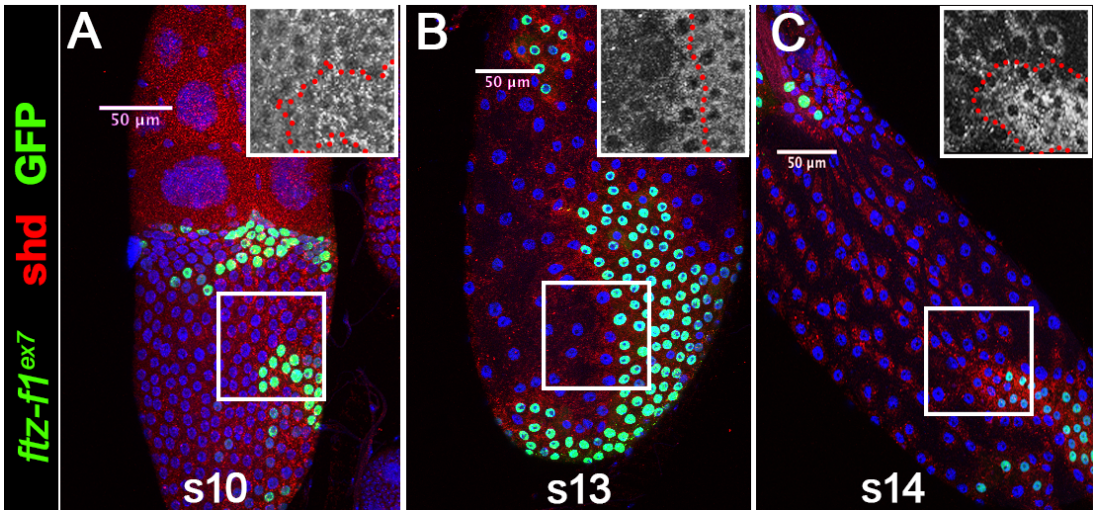

Fig. 4.14. Expression of shade in *ftz-f1*^{ex7} mutant clones.

(A-C) *ftz-f1*^{ex7} mutant follicle cell clones (marked with GFP (green) and outlined in insets)) exhibit expression of Shade protein (red, (white in insets)) at wildtype levels in stage-10 follicles (A), with stage-10 expression levels remaining consistent in stage-13 (B) and stage-14 (C) follicles. The insets are higher magnification of Shade expression (white) in outlined areas. Nuclei are marked by DAPI in blue.

Chapter 5 : Summary of Major Findings & Future Directions

Conserved Role of Steroid Signaling for Drosophila Ovulation

Reminiscent to the role for steroid signaling through the PR in mammalian preovulatory follicles (Bishop et al., 2016; Lydon et al., 1996), we demonstrate that steroid signaling through the ecdysone signaling pathway in *Drosophila* preovulatory follicle cells is important for follicle rupture and ovulation. Our findings illustrate the dynamic expression pattern of the biosynthetic enzyme Shade in the final stages of folliculogenesis, which had never been characterized, and demonstrate its upregulation in stage-14 follicle cells is critical for the synthesis of active 20E to signal follicle rupture and ovulation.

Furthermore, we characterized the expression pattern of the EcR isoforms in these late stages of folliculogenesis and found that ovulatory competency requires the downregulation of both the EcR.A and EcR.B1 isoforms, while expression of EcR.B2 in stage-14 follicle cells is sufficient to rescue follicle rupture defects. These isoforms only differ in their A/B domains at the N-terminal and previous work has shown these different isoforms also exhibit differential expression patterns and roles during development and metamorphosis (Talbot et al., 1993). In addition consistent with our findings, previous work had noted the ability of only EcR.B2 to rescue fertility defects in follicle cells expressing EcR^{DN}, implicating EcR.B2 may be the principal isoform in ovarian follicle cells (Cherbas et al., 2003). These findings also implicate specificity in EcR signaling is regulated in part by the AF1 (activation function 1) found in the A/B domain, which is important for the recruitment and interaction of additional co-activator/regulator proteins. It will be interesting to further investigate what potential co-

activators are required for EcR signaling in ovulation, and additionally what mechanisms EcR.A and EcR.B1 play to inhibit this signaling, potentially via competitive inhibition of endogenous signaling or active recruitment of co-repressors to target genes.

Lastly, our work shows ecdysteroid signaling in preovulatory follicle cells mediates proteolytic activity required for follicle rupture, akin to progesterone signaling in mammals. Ecdysteroidal signaling is not critical for the expression of Oamb or Mmp2, indicating that ecdysone regulates a component upstream of Mmp2 activation prior to rupture, or also potentially it may regulate an additional unidentified protease required for rupture in *Drosophila*. This latter hypothesis is intriguing knowing that in mammals PR signaling is required for expression of metalloproteases *Adamts-1* and *Adam8* in preovulatory follicle cells (Robker et al., 2000; Sriraman et al., 2008), and in *Drosophila* a role for Adam-TS has also been identified in follicle cells during early stages (Ozdowski et al., 2009; Wittes and Schüpbach, 2018), but has yet to be studied in the preovulatory follicle. Additionally, we are in preparation to perform CUT&RUN (cleavage under targets and release using nuclease) (Skene and Henikoff) experiments to identify targets of EcR signaling in preovulatory follicle cells to further investigate the targets of ecdysteroids required for follicle rupture and ovulation.

Characterization of a Third Follicle Cell Transition into Ovulatory Competency

The dynamic changes follicle cells experience is very well studied in early stages of folliculogenesis, however later stages have been mostly ignored. Our work identifies a novel transition for follicle cells in the final stages of folliculogenesis, and demonstrate this transition is essential for ovulatory competency. Our findings indicate this

maturation is comprised of a coordinated upregulation of Hnt and downregulation of Cut and Ttk69 to promote expression of both Oamb and Mmp2 for ovulatory competency. Our results also implicate that additional factors within the follicle cells are also functioning during this transition, thus it will be interesting to further untangle the epistatic relationship governing rupture competency in preovulatory follicles. One of the mammalian homologs of Cut is known as CDP (CCAAT displacement protein), and has been shown to function mainly as a transcriptional repressor through inhibiting the activity of a family of transcription factors known as C/EBPs (CCAAT/enhancer binding proteins) (Ellis J Neufield et al., 1992; Mailly et al., 1996). In mammalian ovaries C/EBP expression is mediated by EGFR/RAS/ERK signaling in ovarian follicle cells, and granulosa cell depletion of C/EBP causes ovulation defects in mice (Fan et al., 2009, 2011). In *Drosophila*, the C/EBP homolog is encoded by the gene *slow border cells* (*slbo*) and is highly expressed in the border cells and required for their migration in mid-oogenesis (Montell et al., 1992). These previous studies also indicate *slbo* is expressed in additional follicle cell subsets such as the posterior follicle cells at mid oogenesis, which also correlates with the timing of EGFR/RAS/ERK signaling required in posterior follicle cells during these stages (Fregoso Lomas et al., 2016; Fregoso Lomas et al., 2013; Fuchs et al., 2012; Montell et al., 1992). However, a further relationship between the two has not been studied. Additionally, during these stages downstream of the EGFR pathway the ETS transcription factor Pointed (Pnt) is also functioning in these follicle cells, and some of our preliminary experiments (data not shown) indicate expression of Pnt in posterior follicle cells in the final stages of folliculogenesis is also critical for follicle rupture and ovulation (Boisclair Lachance et al., 2014; Morimoto et al.,

1996). Thus, it will be exciting to consider if there is a requirement for *slbo* expression in posterior follicle cells throughout the end of oogenesis and investigate its relationship with Cut and signaling through the EGFR/RAS/ERK pathway in the transition into ovulatory competency. Future studies to elucidate the signaling paradigm exhibited in this final transition in *Drosophila* folliculogenesis, could provide us with further evidence of the conservation of signaling mechanisms regulating ovulatory competency across species.

Novel Role for Ftz-f1 Signaling in Follicle Cell Maturation

This work also identifies a novel role for the NR5A nuclear hormone receptor Ftz-f1 in regulating follicle cell maturation and is one of the first studies to establish a *bona fide* role for Ftz-f1 signaling in adult *Drosophila*. We found expression of Ftz-f1 is induced in follicle cells through ecdysone signaling at stage 10B and determined Ftz-f1 is required for follicle cell differentiation into ovulatory competence in the final stages of folliculogenesis. We also are the first to demonstrate the expression of the transcription factor Sim in the ovarian follicle cells, and our work shows it functions as a novel target of Ftz-f1 signaling to regulate follicle cell maturation. In addition to regulating ovulatory competency, our findings indicate Ftz-f1 is important for proper dorsal appendage morphology, a complex process that requires proper cell patterning and coordination of multiple signaling components (Boyle and Berg, 2009; Peters et al., 2013; Tzolovsky et al., 1999; Ward and Berg, 2005). Our data shows the expression pattern of Ftz-f1 protein at stage-10B/11 is weaker in dorsal-anterior follicle cell subsets, thus it would be interesting to further investigate how Ftz-f1 is coordinated within these dorsal anterior

follicle cells to pattern the subsequent morphology of the dorsal appendages. Further studies will also be done to investigate the role of Sim in the preovulatory follicle, as we have shown its expression is robustly re-upregulated in stage 14 follicle cells, and preliminary experiments indicate knockdown of Sim specifically at stage 14 also inhibits follicle rupture and ovulation (data not shown).

Lastly, our work establishes Ftz-f1 signaling is required for follicle cells to progress past stage 11 independent of the GA switch, however we have yet to identify how loss of Ftz-f1 could cause follicle cells to arrest in this stage. One possibility is that Ftz-f1 could be regulating metabolic capacities of follicle cells during this stage through production of Acetyl-CoA, a key molecule for facilitation of the Krebs cycle. Acetyl-CoA is a downstream metabolite of ethanol, which requires the enzymatic activity of multiple enzymes such as; Alcohol dehydrogenase (ADH) and Aldehyde dehydrogenase (ALDH) (Heinstra et al., 1983). Previous work has demonstrated Ftz-f1 binds to an enhancer for the alcohol dehydrogenase gene (*Adh*) and can activate transcription (Ayer and Benyajati, 1992; Ayer et al., 1993b), additionally recent studies have found that loss of ALDH in females disrupts the ability of egg chambers to develop after stage-10 of oogenesis (Elgart et al., 2016). These findings suggest Ftz-f1 could be required for metabolic regulation of follicle cells after stage-10 as a critical mechanism for follicle cell maturation.

Another future direction that will be fascinating to investigate is if Ftz-f1 is required for mediating Juvenile Hormone (JH) signaling in follicle cells. Nearly three decades ago it was shown that JH signaling is required for vitellogenin (Vg) (yolk protein) synthesis and uptake in growing oocytes, and coordination of JH and ecdysone

signaling is required for follicles to mature past stage 10 (Bownes, 1989; Handler and Postlethwait, 1978; Soller et al., 1999). The molecular mechanisms underlying JH activation remain somewhat elusive, however studies have indicated JH might be a ligand for the bHLH-PAS domain transcription factor Methoprene-tolerant (Met) (Ashok et al., 1998; Miura et al., 2005) and interestingly, recent work has shown Ftz-f1 facilitates JH activation of gene expression *in vitro* (Dubrovsky et al., 2011). Future studies to explore the possibility that JH signaling in the ovary is also mediated by Ftz-f1 signaling and its downstream target bHLH-PAS transcription factor Sim could provide more insight into the hormonal regulation of follicle development.

Conserved Role for NR5A Receptor Signaling in Follicle Maturation

A role for the NR5A subfamily of nuclear receptors in ovarian follicle cells for female fertility has been demonstrated in mammals, and now for the first time we have discovered the NR5A *Drosophila* homolog Ftz-f1 is also required within the ovarian follicle cells for follicle cell maturation into ovulatory competence. Furthermore, we have shown that the role of Ftz-f1 in regulating follicle cell differentiation is functionally conserved, with the mammalian homolog SF-1 able to rescue follicle cell maturation defects when Ftz-f1 is disrupted. Our results indicate mSF-1 is also able to restore expression of Sim in these follicle cells, and intriguingly mSF-1 is sufficient to induce Sim expression in follicle cells prematurely at stage-10A, but not any earlier. A role for the mammalian single-minded homolog (SIM) has yet to be investigated in the ovary, and this work highlights the potential for SIM to function downstream of SF-1 to regulate folliculogenesis in mammals. Overall, these findings demonstrate the utility of *Drosophila* as a model to study conserved signaling mechanisms in ovarian follicle cells.

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Zhu, J., Chen, L., Sun, G., and Raikhel, A.S. (2006). The Competence Factor βFtz-F1 Potentiates Ecdysone Receptor Activity via Recruiting a p160/SRC Coactivator. Mol. Cell. Biol. *26*, 9402–9412.

(1999). A Unified Nomenclature System for the Nuclear Receptor Superfamily. Cell *97*, 161–163.

Curriculum Vitae

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EDUCATION

2017- anticipated December 2019 Ph.D. Physiology & Neurobiology
Department of Physiology & Neurobiology, University of Connecticut, Storrs CT

2015-2017 M.S. Physiology & Neurobiology
Department of Physiology & Neurobiology, University of Connecticut, Storrs CT

2010-2014 B.S. Biology
Department of Biology, University of Connecticut, Storrs CT
Honors: *summa cum laude*

RESEARCH EXPERIENCE

2015- Current
Graduate Student
Department of Physiology & Neurobiology
University of Connecticut
Advisor: Jianjun Sun, Ph.D.

My Ph.D. dissertation is focused on characterizing the role of nuclear hormone receptor signaling in *Drosophila* ovulation and folliculogenesis. In particular, I aim to identify how two main families of highly conserved nuclear receptor signaling pathways, steroid & NR5A, regulate molecular mechanisms within the ovarian follicle cells to coordinate follicle maturation and ovulation.

2014-2015
Laboratory Technician
Department of Physiology & Neurobiology
University of Connecticut
Advisor: Jianjun Sun, Ph.D.

As a laboratory technician I investigated how JNK signaling functioned in preovulatory follicle cells to control expression of proteases critical for ovulation in *Drosophila*.

2013-2014
Undergraduate Student
Department of Physiology & Neurobiology

University of Connecticut
Advisor: Jianjun Sun, Ph.D.

During my undergraduate training, I performed deletion mapping assays in *Drosophila* to determine the genes disrupted in recessive female sterile mutants.

2013-2014

Undergraduate Student
Department of Kinesiology, Korey Stringer Institute
University of Connecticut
Advisor: Douglas J. Casa, Ph.D.

During my undergraduate training, I worked within the Korey Stringer Institute to gather data surveying the condition of Athletic Trainer programs throughout public high schools across the nation.

AWARDS & FUNDING

2019

University of Connecticut Graduate Student Mentorship Excellence Award

2018

University of Connecticut Doctoral Student Travel Award
University of Connecticut Doctoral Dissertation fellowship
Physiology and Neurobiology Department Logo Design Winner

2017

Physiology & Neurobiology Doctoral Fellowship

2016

The Genetics Society of America Travel Award

PUBLICATIONS

Knapp, EM., Sun, J (In Preparation). The NR5A nuclear receptor Ftz-F1 promotes follicle maturation and ovulation via bHLH transcription factor Single-minded.

Knapp, EM., Li, W., and Sun, J. (2019). Downregulation of homeodomain protein Cut is essential for follicle maturation and ovulation. Development

Knapp, EM., Deady, LD., and Sun, J. (2018). *Ex vivo* Follicle Rupture and *in situ* Zymography in Drosophila. Bio-Protocol

Knapp, EM., Sun, J. (2017). Steroid signaling in mature follicles is important for Drosophila ovulation. PNAS

PRESENTATIONS AT SCIENTIFIC CONFERENCES

Knapp EM., King T, Sun J, Downregulation of Homeodomain Transcription Factor Cut is Essential for Follicle Maturation and Ovulation. Poster Presentation at Drosophila Research Conference 60th Annual Meeting: March 27th-31st, 2019; Dallas, TX.

Knapp EM., King T, Sun J, Homeodomain Transcription Factor Cut Regulates Competency of Drosophila Follicles. Oral Presentation at 7th Annual UCONN Fly Club: January 16th, 2019; Storrs CT.

Knapp EM & Sun J, The Nuclear Hormone Receptor Ftz-F1 is Required for Follicle Cell Maturation. Oral Presentation at 1st COR^{2}E Facilities Symposium: November 12th, 2018; Storrs, CT.

Knapp EM & Sun J, NR5A-family Nuclear Receptor Hr39/LRH-1 plays conserved roles in follicle maturation and ovulation. Poster Presentation at Society for the Study of Reproduction 51st annual meeting: July 10th-13th, 2018; New Orleans, LA.

Knapp EM & Sun J, The NR5A Nuclear Receptor Hr39 Functions in Both Reproductive Glands and Mature Follicles to Regulate Ovulation. Poster Presentation at Drosophila Research Conference, 59th Annual Meeting: April 11th-15th, 2018; Philadelphia, PA.

Knapp EM., Li, W., Sun J, Steroid Signaling in Mature Follicles is Essential for Drosophila Ovulation. Oral Presentation at Drosophila Research Conference at The Allied Genetics Conference, 57th annual meeting; July 14th, 2016; Orlando, FL.

Knapp EM., Li, W., Sun J, Investigating the Role of Steroid Hormones in Drosophila Ovulation. Oral Presentation at 2nd Annual UCONN Fly Club: May 12th, 2016; Farmington, CT.

PROFESSIONAL MEMBERSHIPS

2014-Current	2017-2018
Genetics Society of America	Society for the Study of Reproduction

TEACHING EXPERIENCE

2016-2019 Lab Instructor
Enhanced Physiology & Neurobiology I (PNB2274)

Department of Physiology & Neurobiology, University of Connecticut.

2015-2016 Lab Instructor
Physiology & Neurobiology I&II (PNB2264-5)
Department of Physiology & Neurobiology, University of Connecticut.

2014 Teaching Assistant/Lab Instructor
Physiology & Neurobiology I&II (PNB2264-5)
Department of Physiology & Neurobiology, University of Connecticut.

2013 Teaching Assistant/Tutor through SASP
Principles of Biology (BIOL1107)
Department of Biology, University of Connecticut.

2012 Teaching Assistant/Tutor through SDS
Evolution and Human Diversity (EEB2202)
Department of Ecology and Evolutionary Biology, University of Connecticut.

MENTORSHIP

2019 Michel Ruis, Young Scholars Program, High school Researcher

2018-current Rebecca Oramas, Graduate Student, Sun Lab, University of Connecticut

2018 Jennifer Lawson, Young Scholars Program, High school Researcher

2017-current Ekaterina Skaritanov, Undergraduate Researcher

2017-current Andrew Beard, Graduate Student, Sun Lab, University of Connecticut

2017-2019 Celina Caetano, Undergraduate Researcher

2017 Halie Ostberg NSF REU student

2015- 2019 Tonya Tucker, Undergraduate Researcher

2014-2017 Timothy King, Undergraduate Researcher

SERVICE

2014-current Judge: Connecticut Invention Convention Annual Competition
2011-2018 Volunteer: Greater Hartford Outdoor Track and Field Invitational
2017 Interviewee for UConn STEM Talk Magazine
2016-2017 Graduate Student panel for First Year Undergraduate Students / R.E.U. students

REFERENCES

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